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Award Number: DAMD17-00-1-0361

TITLE: Breast Cancer Training Program

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CONTRACTING ORGANIZATION: University of Nebraska Medical Center
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REPORT DATE: August 2005

TYPE OF REPORT: Final Addendum

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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I. Key Accomplishments.

Task 1. **We will develop and maintain a Breast Cancer Training Program for graduate students and postdoctoral fellows that will include the following academic programs (programs and activities were detailed in application).** The Breast Cancer Focus Group continues to meet monthly and the Student/Fellow Research Forum continues to meet weekly during the academic year. The 2005 Short Course in Cancer Biology was held May 16-18 and the internationally recognized visiting scientists for this course are listed in Table 1. The Eppley Institute continues to sponsor an outstanding seminar program with a strong emphasis on breast cancer. Speakers on breast cancer in the 2000-05 academic years are listed in Table 2.

Table 1. Visiting Faculty for 2005 Short Course in Cancer Biology –Cellular Responses to DNA Damage and Cancer

Faculty	Institution	Title of Section
Dr. Thomas Kunkel	National Institutes of Health	DNA Replication (IN) Fidelity Causes and Consequences
Dr. Jean Wang	University of California	Coordination of DNA Repair and Cell Death Response
Dr. Wei Yang	National Institutes of Health	Making and Breaking DNA: Lessons from structural Studies
Dr. Jan Hoeijmakers	Erasmus Medical Center, the Netherlands	DNA Damage and Repair: At the Crossroads of Cancer and Aging

Visiting Faculty for 2004 Short Course in Cancer Biology – Metastasis: Biology and Therapeutic Strategies.

Faculty	Institution	Title of Section
Dr. Danny Welch	University of Alabama, Birmingham	Metastasis Controlling Genes
Dr. Ruth Muschel	Children's Hospital of Philadelphia	Mechanisms of Metastasis
Dr. Patricia Steeg	National Institutes of Health	Translational Leads in Breast Cancer Metastasis
Dr. Lynn Matrisian	Vanderbilt University Eppley Visiting Professor of Oncology	What We've Learned from the Matrix Metalloproteinase Inhibitor Clinical Trials

Visiting Faculty for 2003 Short Course in Cancer Biology - Cell Biology of Breast Cancer

Faculty	Institution	Title of Section
Dr. Jeffrey Holt	University of Colorado Health Science Center, Denver, CO	Pathobiology and Genetics of Breast Cancer
Dr. Nita Maihle	Mayo Clinic, Rochester,MN	Growth Factor Regulation of Breast Cancer
Dr. William Mueller	McGill University, Quebec, ON, Canada	Transgenic Models of Breast Cancer
Dr. Mary (Nora) Disis	Washington University, Seattle, WA	Novel Terapeutic for the Teatment of Breast Cancer

Visiting Faculty for 2002 Short Course in Cancer Biology – Cancer Genetics

Faculty	Institution	Title of Section
Dr. Gerard Evan	University of California, San Francisco	Constructing and Deconstructing a Cancer Genetically
Dr. Donna Albertson	University of California, San Francisco	Microarray Based Comparative Genomic Hybridization to Assess Gene Copy Number
Dr. William Kaelin	Harvard University and Howard Hughes Med. Institute	Tumor Suppressor Genes and Gene Based Therapies
Dr. William Bennett	City of Hope, Beckman Research Institute	Cancer Epidemiology

Visiting Faculty for 2001 Short Course in Cancer Biology

Faculty	Institution	Title of Section
Dr. Matthew Mescher	University of Minnesota	T Lymphocyte Response to Tumors
Dr. James Mule	University of Michigan Medical Center	Tumor Vaccines
Dr. Jeffrey Weber	University of Southern California, School of Medicine	Clinical Immunotherapy Trials
Dr. Ellen Vitetta	University of Texas southwestern Medical Center	Monoclonal Antibody Therapies for Cancer

Table 2. Eppley Institute Seminar Speakers on Breast Cancer, 2000-2005

Date	Speaker	Institution	Topic
June 16, 2005	Dr. Shawn Holt	Massey Cancer Center, Medical College of Virginia at Virginia Commonwealth University	Telomeres and Telomerase in Breast Cancer
May 19, 2005	Dr. Lothar Hennighausen	National Institutes of Health	Sharing genetic keys in development and physiology: lessons from blood and milk
May 5, 2005	Dr. Junjie Chen	Mayo Clinic	Cell Cycle Checkpoints & Tumorigenesis
April 28, 2005	Dr. Jack Weber	University of Nebraska - Lincoln	Mutagenesis and Chromosome Engineering in the Mouse
March 3, 2005	Dr. Saraswati Sukumar	Johns Hopkins University School of Medicine	Molecular markers for early detection of breast cancer- from discovery to application
February 10, 2005	Dr. James Trosko	Michigan State University	Role of Human Adult Stem Cells and Cell-Cell Communication in Carcinogenesis: Ignored 'Hallmarks of Cancer'
December 16, 2004	Dr. Thomas Sturgill	University of Virginia	Regulation of ICK, a CDK and MAPK like Protein Kinase
December 2, 2004	Dr. Weidong Wang	National Institutes of Health	The DNA Damage Response Network of Fanconi Anemia and BRCA proteins
November 11.2004	Dr. Linda Schuler	University of Wisconsin	Prolactin and Breast Cancer
October 7, 2004	Dr. Mien-Chie Hung	University of Texas, M.D. Anderson Cancer Center	Novel Signaling Pathways in Cancer Cells and Development of Targeted Cancer Gene Therapy
September 23, 2004	Dr. Andrei Kuzminov	University of Illinois at Urbana-Champaign	Chromosomal lesions: predisposition, mechanisms and repair
July 22, 2004	Dr. Emanuel F Petricoin	FDA-NCI Clinical Proteomics Program, Office of Cellular and Gene Therapies	Clinical Proteomics: Applications at the Bedside
May 24, 2004	Dr Evelyne Sage	CNRS Institut Curie , Centre Universitaire	Multiply damaged sites in DNA : a challenge for cellular repair processes?
May 1, 2004	Dr. Nancy E Davidson	Johns Hopkins University School of Medicine	**Henry Lemon Memorial Lecture** Epigenetic Gene Regulation in Breast Cancer
April 22, 2004	Dr. Michael Stallcup	University of Southern California Los Angeles	Role of protein methylation and protein-protein interactions in transcriptional activation by nuclear receptors and their coactivators
April 8, 2004	Dr. Hallier Rui	Georgetown University, Lombardi Comprehensive Cancer Center	The role of Stat5 in human breast cancer progression
March 25, 2004	Dr. Guo-Min Li	University of Kentucky , Department of Pathology	Dissection of DNA mismatch repair in human cells
March 18, 2004	Dr. Paola Muti	Department of Social & Preventive Medicine University at Buffalo	Endogenous hormones and breast cancer risk and recurrence
February 5, 2004	Dr. Dawn Quelle	University of Iowa	Signaling pathways and partners of ARF
December 11, 2003	Dr. Scott Kaufmann	Mayo Clinic, Oncology Research	Apoptosis and the Response to Anticancer Drugs
November 13, 2003	Dr. Thomas Sutter	W. Harry Feinstone Center for Genomic Research, University of Memphis	Functional Genomic Approaches to Chemical Carcinogenesis and Chemoprevention

Table 2. Continued Eppley Institute Seminar Speakers on Breast Cancer, 2000-2005.

Date	Speaker	Institution	Topic
October 9, 2003	Dr. Jun Qin	Lerner Research Institute, The Cleveland Clinic Foundation	Integrin-Mediated Cell Adhesion Viewed at Three Dimensions
September 25, 2003	Dr. Scott Weed	University of Colorado Health Sciences Center	Cortactin: Integrating Tyrosine Kinase and Actin Cytoskeletal Signaling Pathways
May 1, 2003	Dr. Gail Prins	University of Illinois at Chicago	Estrogenic regulation of steroid receptors, morphogens and signaling pathways during prostate development
April 24, 2003	Dr. Jose Russo	Fox Chase Cancer Center	A new paradigm in the prevention of human breast cancer
April 17, 2003	Dr. Jerry Zambetti	St. Jude Children's Research Hospital	Regulators and mediators of the p53 tumor suppressor pathway.
April 10, 2003	Dr. Chu-Xia Deng	NIH	BRCA1 and Tumorigenesis
March 13, 2003	Dr. Donald Patrick McDonnell	Duke University Medical Center	Nuclear receptor-cofactor interactions: Points of convergence between multiple signaling pathway
February 6, 2003	Dr. Russell Reiter	The University of Texas Health Science Center	Actions of Melatonin
January 16, 2003	Dr. Robert Cardiff	University of California, Davis	Breast Cancer: Lessons from the mouse
November 14, 2002	Dr. Philip Thorpe	UT Southwestern Medical Center	Vascular Targeting Agents for the Treatment of Solid Tumors
November 7, 2002	Dr. Barbara Vonderhaar	NCI	Prolactin in Mammary Development and Tumorgenesis
October 31, 2002	Dr. Al Reynolds	The Vanderbilt-Ingram Cancer Center	p 120-Catenin: Tumor Suppressor or Metastasis Promoter
October 24, 2002	Dr. David Zava	ZRT Laboratory Beaverton, Oregon	Hormonal Imbalance and Cancer Risk: The Estrogen Matrix
October 10, 2002	Dr. Joe W. Gray	UCSF Comprehensive Cancer Center	Genome Evolution in Breast Cancer: Predictive Markers to Therapeutics Targets
September 26, 2002	Dr. Martin Privalsky	University of California at Davis	A Molecular Toggle Switch: Nuclear Hormone Receptors and Transcriptional Regulation
April 24, 2003	Dr. Jose Russo	Fox Chase Cancer Center	A new paradigm in the prevention of human breast cancer
May 9, 2002	Dr. Priscilla Furth	Georgetown Univ.	Hormonal signaling and mammary carcinogenesis
April 18, 2002	Dr. Michael Gould	Univ. Wisconsin	Genetics of mammary cancer susceptibility
March 28, 2002	Dr. Daniel Medina	Baylor College of Medicine	Hormones, aneuploidy and mammary carcinogenesis
March 14, 2002	Dr. Itamar Barash	Volcani Institute, Israel	Stat5, mammary carcinogenesis
February 21, 2002	Dr. Robert Callahan	NCI	Notch, mammary gland development
January 17, 2002	Dr. Larry Brody	NCHGR	BRCA1

Table 2. Continued Eppley Institute Seminar Speakers on Breast Cancer, 2000-2005.			
Date	**Date**	**Date**	**Date**
November 15, 2001	Dr. Dihua Yu	U. Texas, M.D. Anderson	ErbB2 and breast cancer chemoresistance
November 8, 2001	Dr. David Soloman	NCI	Cripto, mammary gland morphogen
October 11, 2001	Dr. Wei Zheng	Vanderbilt Univ.	Breast cancer epidemiology
September 6, 2001	Dr. Gilbert Smith	NCI	Mammary stem cells
May 24, 2001	Dr. V. Craig Jordan	Northwestern University School of Medicine	**Henry Lemon Memorial Lecture**: Development of Antiestrogens for Breast Cancer Treatment and Prevention
April 12, 2001	Dr. Joachim Liehr	Stehlin Foundation	Genotoxic Mechanisms of estrogen Carcinogenesis
April 5, 2001	Dr. Jeffrey Rosen	Baylor College of Medicine	Transgenic and Knockout Mouse Models of Breast Cancer
March 22, 2001	Dr. Kathryn Horwitz	University of Colorado Health Science Center	Mechanisms of Hormonal Resistance in Breast Cancer
October 19, 2000	Dr. Douglas Yee	University of Minnesota	The IGF-I Axes and Breast Cancer
November 8, 2001	Dr. David Soloman	NCI	Cripto, mammary gland morphogen

Task 2. **We will recruit qualified students and fellows to the Breast Cancer Training Program and, through the laboratories of the training faculty, provide a stimulating, comprehensive and multidisciplinary training experience pertaining directly to breast cancer.** 10 predoctoral and 15 postdoctoral trainees have been supported by this training grant during the five years of this award. 7 of the 10 predoctoral trainees and 10 of the 15 predoctoral fellows supported by the award have now completed their training at the Eppley Institute and have moved to postdoctoral positions in internationally recognized laboratories. 5 of the 15 postdoctoral trainees supported by the award remain in training. A summary of the research of each fellow is presented below.

Djuana Harvell, Ph.D.; predoctoral trainee supported in year 01.
Dr. Harvell demonstrated that a 40% restriction of dietary energy consumption inhibits estrogen-induced mammary carcinogenesis in the female ACI rat. This inhibition occurs at a step subsequent to development of focal regions of atypical hyperplasia. Two first author manuscripts were published in the past year (Appendices 1 and 2), and a third has been submitted for publication. Dr. Harvell is now a postdoctoral fellow in the laboratory of Dr. Kate Horwitz at the University of Colorado Health Sciences Center, where she is continuing to study the role of steroid hormones in breast cancer.

Michelle VanLith, Ph.D.; predoctoral trainee supported in year 01.
Dr. VanLith defined the cellular bases of tumor-specific immune responses to MUC-1. She is first author of a manuscript, listed below, that has been accepted for publication. A second first author manuscript has been submitted for publication. Dr. VanLith is currently a postdoctoral fellow in the laboratory of Dr. V. Englehard at the University of Virginia, working in the area of tumor immunology.

Jennifer Brennan, Ph.D.; predoctoral trainee supported in year 01.
Dr. Brennan demonstrated that kinase suppressor of ras (KSR) cycles through the nucleus in a phosphorylation dependent manner. Cellular localization was also impacted by specific interactions with MEK. A first author manuscript detailing this study was published in the *Journal of Biological Chemistry* (Appendix 3). Dr. Brennan is currently a postdoctoral fellow at St. Jude Children's Hospital working in the laboratory of Dr. John Cleveland.

Martin Tochacek, Ph.D.; predoctoral trainee supported in year 02.
Dr. Tochacek mapped several genetic loci that determine susceptibility to estrogen-induced mammary cancer in crosses between the highly susceptible ACI strain and two different resistant rat strains. Dr. Tochacek contributed to one published manuscript (Appendix 4) and two first author manuscripts have been submitted for publication. Dr. Tochacek is currently a postdoctoral fellow at Duke University, working in the laboratory of Dr. Donald McDonnell, studying steroid hormones action and breast cancer.

Kimberly Wielgus; predoctoral trainee supported in year 02.
Ms. Wielgus is working toward the Ph.D. in nursing and is investigating fatigue in patients with advanced stage breast cancer. Although Ms. Wielgus is in the early stages of her dissertation research, her participation in the activities of the Breast Cancer Research Program has enabled her to gain a fundamental understanding of the disease process as well as its genetic and molecular bases. Ms. Wielgus competed successfully for a four-year Scholarship in Cancer Nursing from the American Cancer Society.

Tracy Strecker, Ph.D.; predoctoral trainee in year 03
Mr. Strecker is nearing completion of his doctoral studies and has one manuscript submitted for publication at this time. He is currently working on 2 additional manuscripts. His work focused on the identification of genetic loci involved in estrogen-induced tumorgenesis in ACI and Copenhagen Rats.

Chunhui Yi; predoctoral trainee supported in year 04
Ms. Yi demonstrated that the expression of an extracellular matrix protein fibulin-2 is lower in eight breast cancer cell lines compared to normal cells. She is studying the roles and mechanisms of fibulin-2 in breast cancer growth, invasion and metastasis. Cur rently, her work is focus on verifying the expression of fibulin-2 in patient samples. She is also working on establish fibulin-2 expression in those breast cancer cell lines in order to study how fibulin-2 expression affects cancer growth and invasion. Her work will be presented at the AACR meeting and her preliminary data and data to be collected will contribute to a manuscript describing the functions of fibulin-2 in breast cancer.

Marissa Carstens; predoctoral trainee supported in year 04
Ms. Carstens is working toward her Ph.D. in Pathology and Microbiology. In October of 2003, Marissa received an AACR Scholar- in-Training- Award for her poster presentation at the special conference "Advances in Breast Cancer Research: Genetics, Biology, and Clinical Implications" in Huntington Beach, CA.

Jason Huerta, predoctoral trainee supported in year 04/05
Mr. Huerta's research focuses on defining the genetic bases of susceptibility to estrogen (E2)-induced mammary cancer in the ACI rat model. Previously, his laboratory has mapped seven distinct regions of the rat genome that each harbor one or more genes that influence susceptibility to E2-induced mammary cancer. Mr. Huerta is analyzing the expression of *Cdkn2a* and *c-myc* genes which map to a particular E2-induced susceptibility loci (*Emca1 and 4*) that map to chromosomes 5 and 7. In addition, Mr. Huerta is developing a congenic rat line which carries the cancer resistant allele of *Emca4* from the Brown Norway strain (including the c-*myc* gene) on the cancer prone ACI strain background, so that the impact of *Emca4* on E2-induced mammary carcinogenesis can be evaluated independently of the other susceptibility loci.

Mohamed F. Ali, predoctoral trainee supported in year 05
Mr. Ali is examining the molecular mechanism for increased risk of breast cancer due to a commonly inherited polymorphic mutation (Arg399Gln) in the X-ray cross- complementation protein 1 (Xrcc1). Using isogenic cell lines expressing the wildtype and mutant isoforms of the *Xrcc1* gene, he is testing whether, compared to the wild type, the Xrcc1 (Arg399Gln) mutant protein results in an increased mutation frequency by making errors in base excision repair of estrogen-induced DNA depurination.

Benjamin Xie, M.D., Ph.D.; postdoctoral trainee supported in year 01
Dr. Xie demonstrated that expression of progesterone receptor (PR) is much higher in the focal regions of atypical hyperplasia and mammary carcinoma induced in ACI rats by continuous treatment with estradiol than in normal or hyperplastic mammary glands. These data are included in two published manuscripts. Dr. Xie also demonstrated that expression of Cdkn2a is markedly down-regulated as an early event in estrogen-induced mammary carcinogenesis. A manuscript describing these data is in preparation.

Constance Dooley, Ph.D.; postdoctoral trainee supported in year 01
Dr. Dooley tested the hypothesize that ectopic kinase suppressor of ras (KSR) will inhibit the transformation properties of human cancer cells in vitro and the tumorigenic potential of mammary tissue in vivo. Dr. Dooley successfully generated high-titer recombinant baculovirus for full-length KSR, KSR with two mutated phosphorylation sites, the carboxy terminal half of KSR, the amino terminal half of KSR, and two forms of KSR with reduced or absent activity. A manuscript describing these studies is in preparation. Dr. Dooley recently moved to a new postdoctoral training position at the University of Utah.

David Smith, Ph.D.; postdoctoral trainee supported in year 01
Dr. Smith investigated the regulation of the human *MUC1* gene. *MUC1* has been shown to be up-regulated in many forms of cancer including breast. He performed *in vivo* footprinting experiments to locate the positions of transcription factor binding sites in the promoter region of the *MUC1* gene. Finally, he initiated a translational study in which cDNA array technologies are being used to compare gene expression profiles in primary breast cancers and associated axillary lymph node metastasis.

Beverly Schaffer, Ph.D.; postdoctoral trainee supported in year 02
Dr. Schaffer joined the BCTP in December of 2001. She is generating congenic rat lines in which Brown Norway alleles for *Emca1*, *Emca2* and *Emca3* are carried on the ACI background. We have demonstrated that these *Emca* loci determine susceptibility to estrogen-induced mammary cancer in crosses between the ACI and BN rat strains. The congenic lines will be characterized to define the roles of each *Emca* locus in estrogen-induced mammary carcinogenesis and to fine map each *Emca* locus.

Nicholas Moniaux, Ph.D.; postdoctoral trainee supported in year 02
Dr. Moniaux has cloned and characterized the MUC4 genes from rat and human and is defining the interactions between MUC4 and HER2. He is testing the hypothesis that MUC4/HER2 interactions contribute to pathogenesis of breast cancer.

Adrian Reber, Ph.D.; postdoctoral trainee supported in year 02
Dr. Reber is investigating the role of invariant chain protein (Ii) on MHC class I molecules in different breast cancer cell lines from humans, rats and mice. It has been demonstrated that Ii binds only to folded, peptide free, class I molecules and results in increased cell surface expression of class I. Experiments underway will test the hypothesis that Ii may be used to increase anti tumor immune responses in rat and mouse mammary cancer models.

Scott Stoeger, BS; postdoctoral trainee in year 03
Scott is an MD/PhD student working on understanding how the Kinase Suppressor of Ras (KSR) may play a role in determining cellular sensitivity to chemotherapeutic agents. In addition, he is examining the expression of KSR in a variety of cancer cell lines. He is an author on one manuscript that is submitted for publication.

Lois Beckerbauer, Ph.D.; postdoctoral trainee in year 03
Dr. Beckerbauer continues her training in Dr. Shull's laboratory and recently received an individual postdoctoral fellowship from the DOD BCRP. She is coauthor on Dr. Xie's manuscript that is nearing submission and is first author on a second manuscript nearing submission. She had a poster presentation accepted at the 2003 meeting of the AACR.

Chao Jiang, BS, M.D.; postdoctoral research associate in year 03 and 04
Dr. Jiang is currently studying the roles of the transcription cofactors in estrogen receptor-mediated transcription and tumorigenesis. She has given two presentations at the meeting of Cancer Genetics & Tumor Suppressor Genes at Cold Spring Harbor, New York in August 2002. Dr. Jiang is first author on 2 papers which have been submitted.

Yan Zhang, Ph.D.; postdoctoral trainee supported in year 04
Dr. Zhang has been pursuing her proposed research on the role of various cytochromes P450 isoforms in the metabolism of estradiol to form DNA adducts in relation to the initiation of breast cancer. She has given a presentation at the meeting of the American Association of Cancer Research at Orlando, Florida in March 2004.

Kimberly Hansen, Ph.D.; postdoctoral trainee supported in year 04
Dr. Hansen helped develop several congenic rat lines in which the COP allele at one of four Ept loci has been introgressed onto the ACI background. Dr. Hansen is currently characterizing the response of these congenic rat lines to estrogen-induced mammary carcinogenesis and pituitary tumorigenesis. Dr. Hansen predicts that these congenic rat lines will retain their susceptibility to estrogen-induced mammary carcinogenesis similar to that of the ACI rat, but that estrogen-induced pituitary tumorigenesis will be significantly reduced. These experiments are aimed at examining the following hypothesis: that the tumorigenic actions of administered E2 in the mammary gland and the pituitary gland of the ACI rat are genetically separable. Dr. Hansen is co-first author of two manuscripts currently in preparation for August submission.

Steve Schreiener Ph.D.; postdoctoral trainee supported in year 04
Dr. Schreiner is studying the role of the molecular scaffold KSR1 in regulating cell proliferation and motility. He is also studying interactions between the metastasis suppressor nm23 and KSR1. Dr. Schreiner is co-author on a submitted manuscript that demonstrates the role of KSR1 as a regulator of cell differentiation. He is first author on a manuscript in preparation demonstrating the ability of KSR1 to regulate cell motility and adhesion. He has presented his results at a National meeting on breast cancer in Orlando, FL. He also co-author on a manuscript submitted.

Masato Maeda Ph.D.; postdoctoral trainee supported in year 04
Dr. Maeda is studying the role of cadherin switching in TGF-ß1-mediated epithelial to mesenchymal transition in mammary epithelial cells. He has presented his findings at the BCTP meeting, Mar 14, 2003 Eppley Science Hall, the 43 rd Annual Meeting of American Society for Cell Biology (ASCB), Dec 13-17, 2003 San Francisco, CA, 95 th Annual Meeting of American Association for Cancer Research (AACR) Mar 27-31, 2004 Orlando, FL and Gordon Research Conference (Signaling By Adhesion Receptors) Jun 20-25, 2004 Bristol, RI. He also first-author on a manuscript which has been submitted.

Weihua Tang, Ph.D., postdoctoral trainee support in year 04/05
Dr. Tang investigates the role of the tumor suppressor and transcription cofactor TIP30 (Tat-Interacting Protein 30) in the development of breast cancer. Mutations in the TIP30 gene accelerate tumor progression and increase metastasis potential. In particular, Dr Tang studies the role of TIP30 mutants in mammary epithelial cells and the susceptibility of TIP30-deficient mice to rasH induced mammary tumorigenesis. Furthermore, he evaluates the effects of TIP30 missense mutations on the development of breast cancer. Results of his studies were presented at the AACR conference in Anaheim, CA.

Laurice A. Matulka, postdoctoral trainee support in year 05
Dr. Matulka studies the biology and stem cell features of parity-induced mammary epithelial cells (PI-MECs). In particular, Dr. Matulka is investigating the expression profile of various stem cell markers in this unique mammary epithelial cell population, which is specific for females that have undergone at least one full-term pregnancy. The primary objective of her research is to identify the underlying principles of pregnancy-mediated protection against breast cancer.

Task 3. **We will maintain oversight of the Breast Cancer Training Program to ensure that all progress reports and communications are submitted as required and that the training faculty and trainees fulfill their respective obligations to the program.** All previous progress reports have been submitted as required. All activities associated with the Breast Cancer Training Program, as described in our application, have been organized and will continue beyond the term of the award.

II. Reportable Outcomes.

A. Published Manuscripts (funded trainees are underlined):

Matulka, L.A. and K.-U. Wagner (2005): Models of Breast Cancer. *Drug Discovery Today: Disease Models 2(1):1-6*

Triplett, A.A.; K. Sakamoto; L.A. Matulka; L. Shen; G.H. Smith and K.-U. Wagner (2005): Expression of the Whey Acidic Protein (Wap) is necessary for adequate nourishment of the offspring but not functional differentiation of mammary epithelial cells. *Genesis 43 (1): 1-11*

Jill Pecha, Chao Jiang, Weihua Tang, Kristy Bruck, Kay-Uwe Wagner and Hua Xiao (2005). Loss of *Tip30* rapidly immortalizes murine mammary epithelial cells and leads to ductal hyperplasia in the mammary gland. *Submitted for publication*

Chao Jiang, Mitsuhiro Ito, Hua Xiao et al., mTIP30 deficiency increases susceptibility to tumorigenesis. Cancer Res. 2003. 63(24): 8763-7

Chao Jiang, Robert G. Roeder, Hua Xiao. TIP30 interacts with an ERa-interacting coactivator CIA and regulates c-myc transcription. . J Biol Chem. 2004 25;279(26):27781-9

Carstens MJ, Krempler A, Triplett AA, Van Lohuizen M, Wagner KU. Cell cycle arrest and cell death are controlled by p53-dependent and p53-independent mechanisms in Tsg101-deficient cells. J Biol Chem. 2004 Jun 21 [Epub ahead of print].

Harvell, D.M.E., Buckles, L.K., Gould, K.A., Pennington, K.L., McComb, R.D. and Shull, J.D. Rat Strain Specific Attenuation of Estrogen Action in the Anterior Pituitary Gland by Dietary Energy Restriction. Endocrine 2003 21(2) 175-83.

Brennan, J.A., Volle, D.J., Chaika, O.V. and Lewis, R.E. Phosphorylation regulates the nucleocytoplasmic distribution of kinase suppressor of ras. J. Biol. Chem. 277:5369-5377, 2002.

Harvell, D.M.E., Strecker, T.E., Xie, B., Buckles, L.K., Tochacek, M., McComb, R.D. and Shull, J.D. Diet-gene interactions in estrogen-induced mammary carcinogenesis in the ACI rat. J. Nutrition 131:3087S-3091S, 2001.

Harvell, D.M.E., Strecker, T.E., Xie, B., Pennington, K.L., McComb, R.D. and Shull, J.D. Dietary energy restriction inhibits estrogen-induced mammary, but not pituitary, tumorigenesis in the ACI rat. Carcinogenesis 23:161-169, 2002.

Moniaux, N., Escande, F., Porchet, N., Aubert, J.P. and Batra, S.K. Structural organization and classification of the human mucin genes. Front. Bioscience 6:D1192-1206, 2001.

Shiraga, T., Smith, D., Nuthall, H.N., Hollingsworth, M.A. and Harris, A. Identification of two novel elements involved in human MUC1 gene expression in vivo. Mol. Med. 8:33-41, 2002.

Shull, J.D., Pennington, K.L., Reindl, T.M., Snyder, M.C., Strecker, T.E., Spady, T.J., Tochacek, M. and McComb, R.D. Susceptibility to estrogen-induced mammary cancer segregates as an incompletely dominant phenotype in reciprocal crosses between the ACI and Copenhagen rat strains. Endocrinology 142:5124-5130, 2001.

VanLith, M.L., Kohlgraf, K.G., Sivinski, C.L., Tempero, R., and Hollingsworth, M.A. MUC1-Specific Anti-Tumor Responses: Molecular Requirements for CD4 mediated responses. International Immunology 2002 Aug;14(8):873-82.

B. Meeting Abstracts (funded trainees are underlined)

Weihua Tang, Jill Pecha, Mitsuhiro Ito, Kristy Bruck, Christine M. Eischen and Hua Xiao (2005). TIP30 inhibits B cell proliferation and functions as a tumor suppressor in hematopoietic tissue.. *Symposium, AACR, Anaheim 2005.* Oral presentation.

Mohamed Ali and Eleanor Rogan. DNA Repair Gene XRCCI and the Susceptibility to Breast Cancer. *35th Annual Midwest Student Biomedical Research Forum.*

C. Degrees obtained:

Michelle VanLith, Ph.D.	degree granted August 2001.
Djuana Harvell, Ph.D.	degree granted December 2001.
Jennifer Brennan, Ph.D.	degree granted December 2001.
Martin Tochacek, Ph.D.	degree granted June 2002.

D. Cell lines generated:

B. Xie/J. Shull	normal mammary epithelium, ACI rat
B. Xie/J. Shull	normal mammary epithelium, Copenhagen rat
B. Xie/J. Shull	estrogen-induced mammary carcinoma, ACI rat
C. Jiang/ H. Xiao	cultured these cell lines HepG2-TIP30 and HepG2-TIPM3

E. Related funding:

Kim Wielgus, Scholarship in Cancer Nursing, American Cancer Society, 2002-2006.
Beverly Schaffer received an individual postdoctoral fellowship from the DOD BCRP.
Lois Beckerbauer received an individual postdoctoral fellowship from the DOD BCRP.
Kim Wielgus, Scholarship in Cancer Nursing, American Cancer Society, 2002-2006 and an additional grant for her research studies from NASA.
Scott Stoeger has been awarded a graduate studies assistantship from UNMC.
Masato Maeda has been awarded the Research Fellowship from the Uehara Memorial Foundation 4/1/04 – 3/31/05

F. Employment received:

Djuana Harvell (Ph.D., December 2001) accepted a postdoctoral position in the laboratory of Dr. Kate Horwitz at the University of Colorado Health Science Center. She is studying steroid hormones and breast cancer.

Dr. Michelle VanLith (Ph.D., June 2001) accepted a postdoctoral position in the laboratory of Dr. V. Englehard at the University of Virginia, working in the area of tumor immunology.

Jennifer Brennan (Ph.D., December 2001) accepted a postdoctoral position in the laboratory of Dr. John Cleveland at St. Jude Children's Hospital.

Martin Tochacek (Ph.D., June 2002) accepted a postdoctoral position in the laboratory of Dr. Donald McDonnell at Duke University, working in the area of steroid hormone action.

Kim Wielgus is on schedule to graduate with her Ph.D. next year.

Tracy Strecker has taken a postdoctoral postion at Baylor College of Medicine with Dr. Powell Brown

Benjamin Xie has taken a postdoctoral position at University of Nebraska-Lincoln

Constance Dooley (postdoctoral trainee, 2000-2002) accepted a postdoctoral position in the laboratory of Monica Vetter at the University of Utah.

David Smith (postdoctoral trainee supported in 2001-2002) accepted a position of Instructor in the UNMC Department of Surgery.

Nicholas Moniaux (postdoctoral trainee 2002-2003) accepted a research assistant professorship in the Department of Biochemistry and Molecular Biology at UNMC.

Adrian Reber (postdoctoral trainee supported in year 2001-2002) accepted a position of Post-doctoral Fellow at the University of Georgia in the Department of Large Animal Medicine.

Lois Beckerbauer is no longer at UNMC.

Chao Jiang (postdoctoral trainee supported in year 2002 & 2004 of the grant) accepted a position of Research Assistant in the UNMC Department of Biochemistry with Dr. Hua Xiao.

Kim Hansen is an Assistant Professor at Methodist College of Nursing

Steve Schreiner (postdoctoral trainee supported in year 2004 of the grant) accepted the position of Assistant Professor at the University of Nebraska at Omaha.

Masato Maeda completed his postdoctoral training and moved back to Japan.

ELSEVIER
DRUG DISCOVERY TODAY: DISEASE MODELS
Cancer

Models of breast cancer

Laurice A. Matulka, Kay-Uwe Wagner*

Eppley Institute for Research in Cancer and Allied Diseases and the Department of Pathology and Microbiology, University of Nebraska Medical Center, 986805 Nebraska Medical Center, Omaha, NE 68198-6805, USA

The intention of this review is to provide a succinct overview about the availability and relevance of the major categories of mouse models for breast cancer. The review concentrates on the latest achievements in developing genetically engineered mice with conditional knockout alleles or models that allow the inducible expression of oncogenes in mammary epithelial cells. In particular, we discuss the applicability of these models for drug target validation. Furthermore, we critically evaluate experimental designs for modeling cancer prevention and therapeutic intervention by genetic means *in vivo*.

Section Editors:
Vincent Goffin – INSERM, Faculté de Médecine Necker, Paris, France
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Introduction

The pharmaceutical industry uses model organisms, in particular the laboratory mouse (*Mus musculus*), for preclinical studies and toxicity testing. Besides testing drugs to ascertain their safety, researchers are now seeking animal models that target particular pathways and authentically replicate specific human diseases such as breast cancer. Experts repeatedly emphasize that inadequate animal models are one of the major hurdles in drug discovery and development. Identifying models for diseases like breast cancer, therefore, is a priority for many laboratories. The majority of human ailments are, however, polygenic or multifactorial diseases. Breast cancer is no exception in this regard because individual cases differ significantly in their morphology, histopathology, dependence on endogenous growth factors, their activation/inactivation of specific genes and, most of all, in their clinical outcome. Hence, there cannot be only one model for breast cancer but rather a myriad of models, each being unique to a different subtype or a particular aspect of the disease.

Main categories of breast cancer models

Wild-type mice do not develop mammary tumors during their lifetime unless they are inbred strains that carry the mouse mammary tumor virus or other selected mutations. In all types of mouse models, mutations are introduced to initiate and speed up neoplastic transformation. Currently available mouse models for human breast cancer can be categorized into three main groups: (a) xenograft models; (b) chemically induced, virally induced or ionizing radiation-induced models; and (c) genetically engineered mice (GEM) such as transgenics and knockouts. More complex models rely on a combination of particular methodologies used to generate these three main types of mammary cancer models. For example, transgenic mice are being treated with ionizing radiation or chemical carcinogens to accelerate mammary tumorigenesis. Animal models from each of the main groups have their advantages and shortcomings that we discussed in more detail in a recent commentary [1].

Xenograft models

Conventional xenograft models are still widely used in preclinical trials. For a list of available breast cancer cell lines used in xenograft modeling, please refer to a recent article by Kim *et al.* [2]. Xenograft models are relatively inexpensive, easy to generate, and tumors appear after a relatively short

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1740-6757/$ © 2005 Published by Elsevier Ltd. DOI: 10.1016/j.ddmod.2005.05.008

latency. Unlike the majority of neoplastic lesions from chemically induced mouse models or GEMs, several human breast cancer cell lines are estrogen receptor (ERα)-positive. Unencumbered by intellectual property concerns, they are currently indispensable for preclinical testing of inhibitors of steroid receptor signaling and drug resistance studies. Nevertheless, these models are generally poor predictors of response to therapy in humans. Virtually nothing is known about the inciting genetic events in the parental tumors, from which these cell lines were derived. Therefore, xenograft models are less useful for proof-of-principle tests for molecularly targeted therapies. Also, it is unreasonable to assume that the genome of these cell lines is stable. In fact, additional mutations and cell selection (genetic drift) frequently occur *in vitro* under variable culture conditions. In addition, doubts as to their actual tumor of origin are factors that question the validity of such models [2]. It is surprising to see many breast cancer studies still using MDA-MB-435 cells (402 published articles in 2004 alone[1]), although it has been repeatedly shown that these cells and their derivatives express melanoma markers [3,4]. This issue is currently passionately debated. Because some other breast cancer cell lines are also suggested to express melanocyte-specific markers [5], it would be interesting to see how many primary tumors actually express these markers and whether cell culture conditions artificially amplify subtypes of cancer cells expressing melanoma markers.

A continuous need for chemically induced breast cancer models

Since the 1940s, many research laboratories have been utilizing chemical carcinogens, in particular polycyclic hydrocarbons (e.g. DMBA) and alkylating agents (e.g. MNU, ENU), to study mammary tumorigenesis in mice. Early studies demonstrated that there are strain differences in the susceptibility to particular agents. A comprehensive review by Medina and Thompson [6] describes in detail the effects of particular carcinogens on specific molecular alterations as well as the role of hormones and dietary factors as modulators for chemically induced mammary tumorigenesis.

Because we are now able to engineer mutations at precise locations within the mouse genome (see next paragraph), chemically induced tumor models seem to be outdated to many researchers in our field. It is, however, misleading to assume that only technological advances determine the superiority of one model over another. This is certainly not the case. For instance, like in humans, a full-term pregnancy significantly reduces the incidence of mammary tumorigenesis in chemically induced breast cancer models [7]. In the vast majority of transgenic breast cancer models generated over the last two decades, however, pregnancy considerably shortens the latency of mammary tumorigenesis. Therefore, many genetically engineered mice might be suitable to study particular aspects of pregnancy-associated mammary tumorigenesis but they are unable to recapitulate the long-term protective effects of a full-term pregnancy on breast cancer. Although this phenomenon is one of the best-studied epidemiological findings on breast cancer in human populations, the cellular and molecular basis for this observation has not been identified. In conclusion, to study the protective effect of pregnancy on breast cancer, chemically induced models are currently highly relevant. For more information on this subject, please refer to the summary report of the 2003 workshop on Early Reproductive Events and Breast Cancer (http://www.nci.nih.gov/cancerinfo/ere).

Genetically engineered mice (GEMs) for modeling breast cancer

Transgenic mice that express oncogenes under the mammary tumor virus long terminal repeat (MMTV-LTR) or other mammary-specific promoters such as the whey acidic protein gene (*Wap*) were the first generation of GEMs for modeling breast cancer. Since the pioneering work conducted by Leder and co-workers 20 years ago [8], hundreds of transgenic strains have been generated to test the biological relevance of several oncogenic pathways for the initiation of neoplastic transformation of mammary epithelial cells. An entire edition of the journal *Oncogene* published in January 2000 was dedicated to review some of the paramount breast cancer models. In addition, the consensus report of the Annapolis Meeting highlighted individual histopathological features present in the first generation of GEMs [9]. The most important lesson that transgenic mice taught us was that tumorigenesis is indeed a multistep process involving different signaling pathways. Again, Leder and co-workers led the way by demonstrating first that two oncogenes can act in synergism to accelerate neoplastic transformation [10].

Conventional knockouts with targeted mutations of tumor susceptibility genes represent the second generation of GEMs. For example, gene targeting since 1992 has generated more than 20 different germline mutations of the Trp53 gene alone. However, the tumor spectrum in these mice often differs from humans with inherited mutations. For instance, most p53-deficient mice succumb to lymphoid neoplasia before they develop carcinomas. The transplantation of mammary epithelia from knockout donors containing multipotent stem cells into epithelia-divested mammary fat pads of wild-type recipient mice is an elegant technique to bypass the shortcomings of conventional knockouts and to establish mice lacking tumor suppressor genes specifically in the mammary gland [11]. The introduction of missense mutations into tumor suppressor genes (i.e. the generation of knock-in mutants) is another approach to more faithfully mimic human genetic diseases. For example, mice heterozygous for a p53 mutant allele (R175H substitution) differed from

[1] PubMed search for 'MDA-MB-435 AND 2004'.

conventional p53 knockout mice in their tumor spectrum. They exhibited a significant increase in the number of carcinomas and a slight decrease in the number of lymphomas [12].

Thus far, GEMs of the first and second generation are used primarily to study the biological function of genes during normal development and tumorigenesis. The US Food and Drug Administration is considering altering the guidelines on preclinical testing for the carcinogenicity of pharmaceuticals and specific strains are now being used in selective chemoprevention and chemotherapy trials (for examples, please refer to a more comprehensive review by Van Dyke and Jacks [13]).

GEMs that allow an inducible overexpression of oncogenes

The first and the second generation of GEMs allow us to examine whether oncogenes or tumor suppressor genes are involved in tumor initiation. For the development of cancer drugs, in particular for drug target validation, it would be essential to know whether cancer-initiating or cancer-promoting genetic alterations are essential for the survival of neoplastic cells within progressing lesions. The scientific challenge of determining whether a multistage cancer process is reversible fueled the development of novel mouse models that overexpress oncogenes in a temporally and spatially controlled manner. There are several inducible systems that can be employed to express transgenes conditionally *in vivo* (for an overview on available techniques, please refer to a review by Mills [14]). Thus far, only tetracycline (tet)-based systems have been utilized successfully to regulate the expression of oncogenes in an inducible fashion in the mammary gland and other epithelial cell types. In a nutshell, a tetracycline-transactivator system [15] has three components: (I) a transgene that directs the expression of the tet-responsive transactivator protein (tTA) to a particular cell type, (II) a second transgene that controls the expression of the oncogene using the tet-operon linked to a minimal promoter derived from the human cytomegalovirus immediate early gene 1 (tet-op) and (III) a tetracycline derivative such as doxycycline (Fig. 1). The transactivator protein is a hybrid composed of the tetracycline repressor protein from *E. coli* transposon TN10 fused to the viral protein 16 (VP16) activation domain from the herpes simplex virus. The transgene expression of the oncogene under control of the tet-op sequence is suppressed by the administration of tetracycline (Tet-Off system). A mutated tetracycline repressor domain was utilized to generate a reverse transactivator (rtTA or Tet-On system) [16]. In this system, the rtTA binds operator sequences and activates the oncogenic transgene only when tetracycline is administered to the animal. While on sabatical at the laboratory of Peter Gruss at the Max-Planck-Institute in Goettingen (Germany), Priscilla Furth (University of Maryland) and Lothar Hennighausen (NIDDK, NIH) were first to adapt the tet-inducible system to transgenic mice [17]. Subsequently, these researchers developed transgenic mice that express the transactivator under the LTR of the mouse mammary tumor virus (MMTV-tTA; JAX® Stock #002618) [18]. These mice were bred to a transgenic strain carrying the simian virus 40 (SV40) T antigen (TAg)-coding sequence

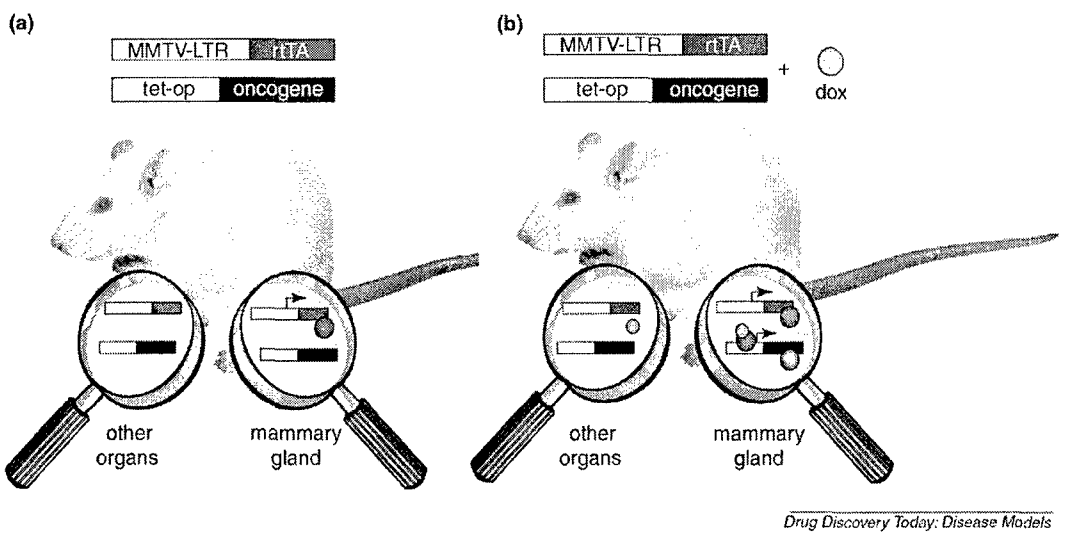

Figure 1. The bi-transgenic tetracycline-inducible system (Tet-On) allows a temporally and spatially controlled expression of oncogenes in the developing mammary gland of genetically engineered mice. (a) The tissue specificity of the tet-inducible system is mediated by regulatory elements (here the MMTV-LTR) that control the targeted expression of the reverse transactivator protein (rtTA). (b) The administration of a tetracycline analog (doxycycline, dox) regulates the binding of the transactivator protein to operator sequences within the promoter of the transgene, which regulates the expression of the oncogene. Withdrawal of doxycycline leads to a deactivation of the tet-op-driven oncogene.

linked to a tet-op promoter [19]. This animal model and the resulting landmark publication in the journal *Science* provided for the first time experimental evidence suggesting that tumorigenesis is reversible at an early stage of neoplastic transformation and that progressing tumor cells can become independent from the tumor-initiating event. Unfortunately, the expression of the transactivator protein in this particular MMTV-tTA strain exhibited less expression in the mammary gland as compared with other organs, and the analysis of tumorigenesis remained restricted to the salivary gland.

Six years later, the laboratory of Lewis Chodosh (University of Pennsylvania) generated a mouse strain that expresses the reverse transactivator under the control of the MMTV-LTR (MMTV-rtTA) [20]. Its efficient expression in the mammary gland was determined by reporter genes (luciferase, LacZ) fused to the minimal promoter/tet-op sequences. Although reporter gene expression and activity could be controlled in the mammary gland, its activation was also detected in several other tissues, including the salivary gland, thymus and seminal vesicle. Since its inauguration, this strain has been utilized in several experiments to generate tumor models that overexpress various oncogenes such as *ErbB2* [21], *Wnt-1* [22] and *c-Myc* [23]. Interestingly, the downregulation of *ErbB2* resulted in reversible pulmonary metastasis, whereas a sustained regression of *c-Myc*-induced mammary lesions following brief or prolonged *c-Myc* inactivation was not observed. These observations might suggest that, unlike *c-Myc*, targeting *ErbB2* could be therapeutically relevant for advanced stages of breast cancer. Regarding drug target validation, the tet-inducible system is clearly superior to the first generation of transgenic tumor models. Unfortunately, four years after their introduction, these tool mice are still not available to the broad scientific community through non-for-profit distributors such as the Jackson Laboratory or the Mouse Model for Human Cancer Consortium (MMHCC).

Another interesting technology to study signal transduction in transgenic breast cancer models was published recently by the laboratory of Jeff Rosen (Baylor College of Medicine). A drug-mediated dimerization of the fibroblast growth factor receptor 1 (Fgfr1), which acts independent of its natural ligand, induced the formation of mammary tumors [24]. Although this system does not affect the transcriptional regulation of the oncogene, it modulates signal transduction pathways through protein–protein interaction. In this regard, such models might better validate drugs in a pharmacological setting, in which small molecule inhibitors affect only particular functions of a protein. Additional functions of a protein, including a role as a scaffold for signal transduction, might not be affected by this approach.

GEMs with conditional knockout alleles

Both inducible systems (i.e. Tet-On and ligand-dimerization) described above require that inducible ligands are administered continuously to the animals to induce tumor formation. Also, current experimental designs that utilized these technologies only studied the importance of transforming oncogenes in progressing tumors. Thus far, they did not manipulate tumor suppressor proteins through, for example, the overexpression of dominant negative molecules or antisense constructs. Also, these techniques are not designed to deregulate the expression of downstream mediators or effectors of tet-inducible oncogenes. These limitations can be overcome in GEMs that carry conditional knockout alleles.

Conditional knockout mice on the basis of the Cre-lox technology were originally developed to bypass embryonic lethality observed in several conventional knockout mice. This includes mouse models that lack tumor suppressor genes implicated in breast carcinogenesis such as *Brca1*. Cre is a site-specific recombinase, which allows for a cell-type-specific deletion of floxed target genes in genetically engineered mice (Fig. 2). Again, it was the pioneering work of the laboratory of Lothar Hennighausen (NIH), which generated the first transgenic mouse strains (Wap-Cre and MMTV-Cre mice; JAX® Stock #003551-003553) that allow a mammary epithelial-specific deletion of genes at various stages during mammogenesis [25]. Both transgenic lines were employed shortly thereafter by the groups of Chuxia Deng and Lothar Hennighausen (both investigators were postdoctoral fellows at the Leder laboratory) to generate the first mouse model for hereditary human breast cancer by deleting the *Brca1* gene conditionally in mammary epithelial cells [26]. These

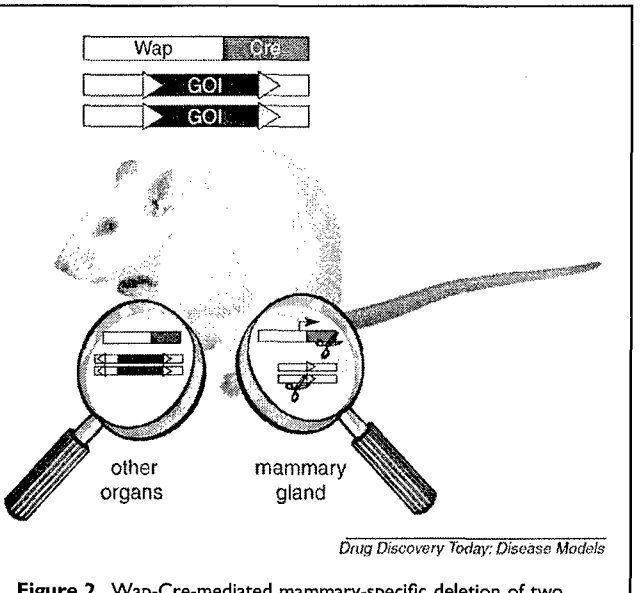

Figure 2. Wap-Cre-mediated mammary-specific deletion of two alleles of a gene of interest (GOI). The promoter of the *whey acidic protein* gene (*Wap*) targets the expression of the Cre recombinase ('molecular scissors') to the mammary gland. Cre recombinase binds to *loxP* (locus of X-ing over) recognition sites (yellow triangles). Cre is able to mediate the recombination and excision of DNA fragments located between two directly oriented *loxP* sites, leaving one *loxP* site in the chromosome.

ground-breaking experiments also addressed the genetic interaction between two tumor suppressor proteins (Brca1 and p53) during the initiation and promotion of mammary tumorigenesis. Since the Wap-Cre and MMTV-Cre mice became available in 1999 through various non-for-profit distributors, these strains were employed by several laboratories to generate a growing number of mouse models (more than 50 to date) that lack a variety of proteins regulating mammogenesis such as hormone receptors, signal transducers, as well as regulators for cell cycle and apoptosis. These conditional knockout models also taught us that some suggested breast cancer susceptibility genes are not involved in neoplastic transformation as previously reported from cell culture studies [27].

Conclusions

Originally designed to bypass embryonic lethality of conventional knockouts, Cre-lox-based conditional models are much more versatile. They can be utilized in several very diverse experimental settings. For example, conventional knockouts often cause pleiotropic effects. In particular, the ablation of hormones, their receptors, or additional downstream signal transducers frequently cause infertility or reduced fertility in females that, in turn, indirectly affect ductal elongation and mammary epithelial specification. Hence, conditional knockout models are helpful to separate systemic effects from cell intrinsic functions of genes. In addition to studying the function of genes during normal development, many conditional mutants will become important for breast cancer research. It is currently the standard to cross conventional knockouts (e.g. cyclin $D1^{-/-}$, $ER\alpha^{-/-}$, $Stat5^{-/-}$) into a variety of transgenic strains overexpressing different oncogenes to assess the effects of a gene ablation on mammary tumorigenesis [28–32]. Because tumorigenesis was absent or delayed in these complex models, the authors concluded that the functional inhibition of these targets might serve a suitable strategy for *therapy* in human lesions that express corresponding oncogenes [30]. Clearly, this is a premature conclusion. Because these females never developed mammary cancer, they might serve as models for cancer *prevention*, but these studies do not allow for conclusions about targeted cancer *therapy*. Two things are essential to model therapeutic intervention: (a) animal models need to develop progressing tumors and (b) the therapeutic target protein has to be expressed in neoplastic cells. Conditional knockout mice can be utilized to better model chemotherapy by genetic means. In its simplest experimental design, the Cre-lox technology can determine whether the genetic ablation of a particular gene is relevant for chemoprevention (deletion before tumor onset) and therapy (deletion in preneoplastic, neoplastic, or metastatic cells). It will be interesting to see how many of the suggested therapeutic targets will be validated when they are knocked out specifically in neoplastic cells using the Cre-lox technology. Whether one uses conventional and conditional knockout models to test the efficacy of particular proteins or pathways as molecular targets for prevention and therapy, the effect of the ablation of a gene on the expression of the oncogene needs to be addressed prior to the study.

Acknowledgements

K-U.W. is supported by the Susan G. Komen Breast Cancer Foundation (BCTR0402956) and a Public Health Service grant (CA93797) from the National Cancer Institute. L.A.M. received financial support from the UNMC Breast Cancer Research Training Program.

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ARTICLE

Expression of the Whey Acidic Protein (Wap) Is Necessary for Adequate Nourishment of the Offspring But Not Functional Differentiation of Mammary Epithelial Cells

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Received 23 May 2005; Accepted 4 June 2005

Summary: Whey acidic protein (WAP) is the principal whey protein found in rodent milk, which contains a cysteine-rich motif identified in some protease inhibitors and proteins involved in tissue modeling. The expression of the *Wap* gene, which is principally restricted to the mammary gland, increases more than 1,000-fold around mid-pregnancy. To determine whether the expression of this major milk protein gene is a prerequisite for functional differentiation of mammary epithelial cells, we generated conventional knockout mice lacking two alleles of the *Wap* gene. Wap-deficient females gave birth to normal litter sizes and, initially, produced enough milk to sustain the offspring. The histological analysis of postpartum mammary glands from knockout dams does not reveal striking phenotypic abnormalities. This suggests that the expression of the *Wap* gene is not required for alveolar specification and functional differentiation. In addition, we found that Wap is dispensable as a protease inhibitor to maintain the stability of secretory proteins in the milk. Nevertheless, a significant number of litters thrived poorly on Wap-deficient dams, in particular during the second half of lactation. This observation suggests that Wap may be essential for the adequate nourishment of the growing young, which triple in size within the first 10 days of lactation. Important implications of these findings for the use of *Wap* as a marker for advanced differentiation of mammary epithelial cells and the biology of pluripotent progenitors are discussed in the final section. genesis 43:1–11, 2005. © 2005 Wiley-Liss, Inc.

Key words: whey acidic protein (WAP); mammary gland; differentiation; gene targeting

INTRODUCTION

The proliferation and differentiation of mammary epithelial cells is controlled by the synergistic action of peptide and steroid hormones as well as local growth factors (Hennighausen *et al.*, 1998, 2001; Topper *et al.*, 1980). Loss-of-function studies in mouse models have demonstrated that prolactin (PRL) signaling though the Jak2/Stat5 pathway plays a central role in this process. PRL (Horseman *et al.*, 1997), the PRL receptor (PRL-R) (Ormandy *et al.*, 1997), the Janus kinase 2 (Jak2) (Shillingford *et al.*, 2002; Wagner *et al.*, 2004), and the signal transducers and activators of transcription 5 (both Stat5a and Stat5b) (Cui *et al.*, 2004; Liu *et al.*, 1997; Teglund *et al.*, 1998) exhibit an unexpected level of specificity during mammogenesis. Indispensable functions of these proteins in the mammary gland are restricted to alveolar proliferation and differentiation during pregnancy. The PRL signaling cascade has many transcriptional targets; among them, genes that regulate cell proliferation and cell adhesion as well as milk protein genes (Gass *et al.*, 2003). The expression of milk protein genes, however, varies slightly between caseins and whey proteins. In mice, casein transcription increases rather early during pregnancy, whereas high levels of expression of the whey acidic protein (Wap) and α-lactalbumin are restricted to the last phase of pregnancy (Pittius *et al.*, 1988; Robinson *et al.*, 1995). The PRL-induced activation and nuclear localization of Stat5a seems to be imperative

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Contract grant sponsor: Susan G. Komen Breast Cancer Foundation; Contract grant number: BCTR0402956; Contract grant sponsor: National Cancer Institute; Contract grant number: CA93797; Contract grant sponsor: Nebraska Cancer and Smoking Disease Research Program; Contract grant number: NE DHHS LB595 (to K.U.W.).
Published online in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/gene.20149

for the transcriptional activation of the *Wap* gene, but not for the expression of β-*casein* (Liu *et al.*, 1997). Stat5 target sequences are part of composite response elements within the *Wap* gene promoter. These elements encompass Stat5, nuclear factor 1, and glucocorticoid receptor binding sites that confer a mammary gland-specific and hormonally regulated expression of *Wap* (Li *et al.*, 1995). Beside hormones and local growth factors, a proper expression of *Wap* also requires cell-to-cell contact and the correct three-dimensional structure of an alveolus (Chen *et al.*, 1989). Wap, therefore, is commonly applied as an advanced differentiation marker for mammary epithelial cells, and its upregulation heralds the appearance of alveolar cells secreting milk (Robinson *et al.*, 1995).

Wap is a major protein in the whey fraction of milk from rodents (Campbell *et al.*, 1984; Hennighausen *et al.*, 1982), rabbits (Devinoy *et al.*, 1988), pigs (Simpson *et al.*, 1998), camels (Beg *et al.*, 1986), and marsupials (Simpson *et al.*, 2000). It has been shown recently that the human genome also contains a *Wap* gene sequence with critical point mutations within the coding region, including the ATG start codon, that may account for the absence of Wap from human milk (Rival-Gervier *et al.*, 2003). The *Wap* transcript was first cloned by Hennighausen and Sippel (1982) from lactation-specific mRNAs of mouse mammary glands. Based on the sequence, the authors determined that *Wap* is a member of the family of four-disulfide-core (4-DSC) proteins that show a cysteine pattern, which is very similar to the hypothalamic carrier protein neurophysin (i.e., a component of pro-oxytocin and pro-vasopressin). The following identification of genes in human and mice, which encode proteins with a Wap-like 4-DSC domain (i.e., the "WAP motif"), led to their classification as the *Wap* gene family. Unlike Wap, which is exclusively expressed in the late-pregnant and lactating mammary gland, many of the WAP-related proteins are present in a variety of tissues, in which they are suggested to function as protease inhibitors. For further information about individual members of the *Wap* gene family please refer to a recent comprehensive review by Simpson and Nicholas (2002).

Despite numerous studies on the transcriptional regulation of the *Wap* gene during pregnancy and lactation, the biological function(s) of Wap in vivo remain, at least in part, elusive. The overexpression of *Wap* under its native regulatory elements, or the mouse mammary tumor virus long terminal repeat (MMTV-LTR), or the ubiquitously active chicken β-*actin* promoter led to premature differentiation and impaired alveolar development in transgenic mice and pigs (Burdon *et al.*, 1991; Hennighausen *et al.*, 1994; Nukumi *et al.*, 2004; Shamay *et al.*, 1992). These studies emphasize the notion that the correct temporal and spatial regulation of *Wap* is tightly linked to advanced differentiation and that Wap might play an important role in this process. Thus far, a conventional knockout model has not been available to test this assumption. Ludwig *et al.* (2001) recently generated the first mouse model with a genetically engineered *Wap* allele by targeting the Cre recombinase coding region into the 5-prime untranslated region (UTR) of the *Wap* locus. This genetic alteration of the *Wap* locus had apparently no phenotypic consequences. A reduction of the *Wap* mRNA or the Wap protein, however, had not been demonstrated in this animal model.

In this report, we describe the generation and analysis of conventional Wap knockout mice. Our main objective was to determine 1) whether the expression of this major milk protein is a prerequisite for functional differentiation of mammary epithelial cells; 2) whether Wap is an essential nutritional component of mouse milk to support the growth of the suckling offspring; and 3) whether Wap has a biologically relevant function as a protease inhibitor, as suggested from its structural analysis. Homozygous Wap knockouts developed normally until adulthood. Mutant females gave birth to normal litter sizes and, initially, produced enough milk to nurse the offspring. The histological analysis of postpartum mammary glands from knockout dams did not reveal striking phenotypic abnormalities associated with Wap deficiency. Our observations suggest that the expression of the whey acidic protein is not required for functional differentiation of mammary epithelial cells. Important implications for the use of the transcriptional activity of *Wap* as a marker for advanced differentiation of mammary epithelial cells and the biology of pluripotent progenitors are discussed in the final section. Despite the absence of a phenotype on the histological level, pups suckling on Wap-deficient dams, regardless of their genotype, were malnourished, and many of them could not be weaned after a normal lactation period of about 21–25 days postpartum. Hence, the secretion of the whey acidic protein into the milk, in particular during the second half of lactation, is important for adequate nourishment to meet the needs of the growing young. Finally, our experiments show that Wap is not an essential protease inhibitor to maintain the stability of other secretory proteins in the milk.

RESULTS

Targeted Deletion of the *Wap* Gene by Homologous Recombination

To study the in vivo function of the whey acidic protein, we replaced the first coding exon of the *Wap* locus with a neomycin resistance gene (Fig. 1A). In brief, a BAC clone encompassing the *Wap* locus was isolated from a mouse 129SvJ genomic library (Incyte Genomics, Wilmington, DE). Two contiguous DNA fragments harboring the entire *Wap* gene and more than 4 kb of flanking sequence on either end were subcloned into pZErO (Invitrogen, La Jolla, CA). A *Kpn*1/*Eco*R1 fragment harboring the entire coding region of *Wap* was sequenced (GenBank AY923114). A comparison of the 129SvJ sequence to a previous release of the *Wap* locus from the GR strain (U38816) revealed substantial inconsistencies within noncoding regions (introns and 3′ flanking

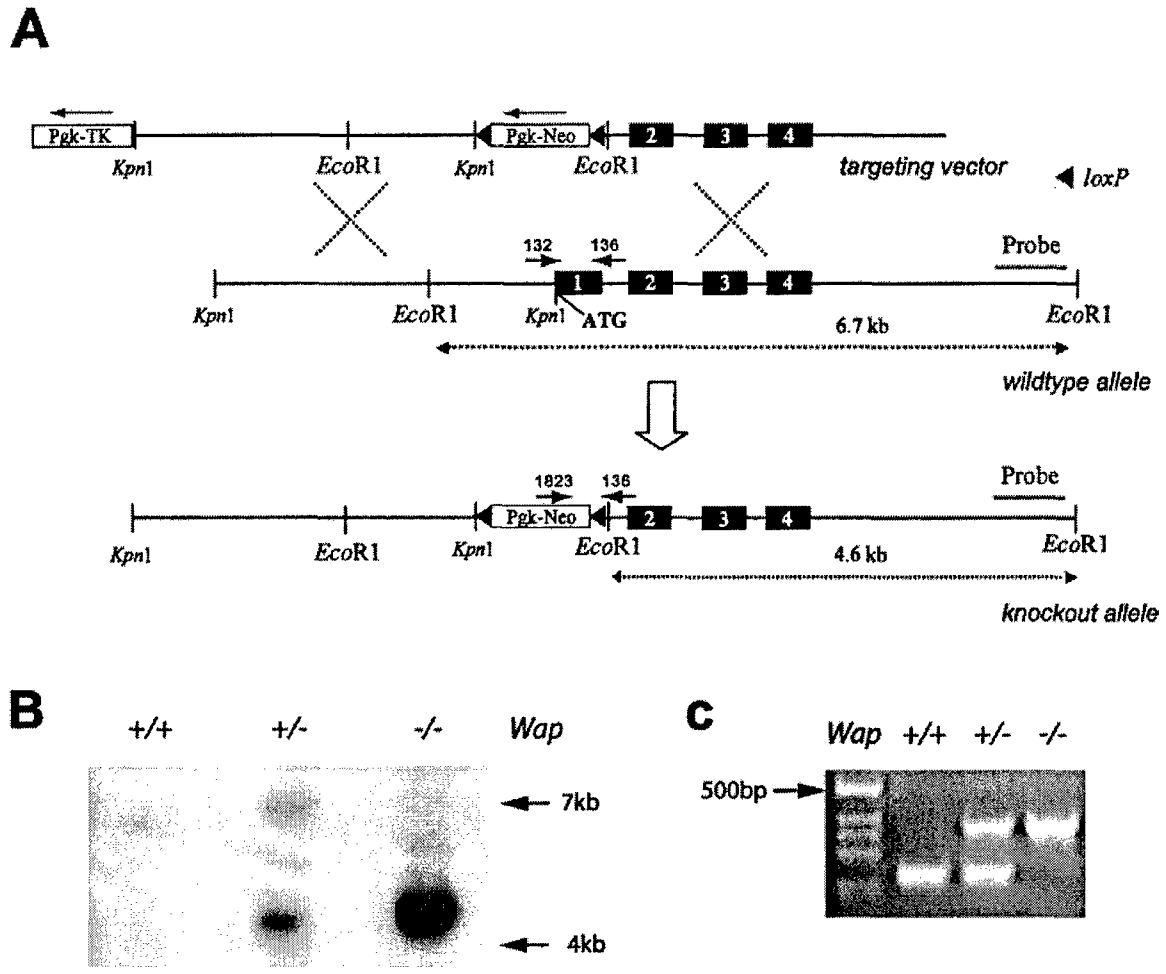


FIG. 1. Targeted deletion of the *whey acidic protein* (*Wap*) gene. **A:** Strategy to replace the first coding exon of *Wap* with a floxed PGK-neomycin resistance gene using homologous recombination in embryonic stem cells. **B:** Southern blot analysis using an *Eco*R1 restriction digest of genomic DNA in combination with a 3-prime external probe (see **A** for location) to verify the presence of the wildtype and/or knockout alleles in heterozygous (+/−) and homozygous mutants (−/−) as well as wildtype controls (+/+). **C:** PCR analysis on genomic DNA of tail biopsies for genotyping of Wap-deficient mice and their heterozygous and homozygous wildtype controls. Arrows indicate the location of the PCR primers (132/136/1823) in **A**.

region). More important, we found three single nucleotide polymorphisms within the coding region of exons 1, 2, and 3 that resulted in amino acid substitutions (R11L, Q35P, and T90M). Two of the three polymorphisms (11 and 35) are consistent with sequence variations that have been documented previously (see Swiss-Prot, P01173). A targeting vector was constructed by replacing the first coding exon and subsequent intron/exon junction with a PGK-neomycin (PGK-neo) selectable marker flanked by *lox*P sites. The PGK-tk cassette was used for negative selection against random integration events. The *Wap* promoter and 5-prime UTR were not genetically modified to allow a further manipulation of the targeted *Wap* locus in embryonic stem (ES) cells. For instance, targeted ES cells can be used for the site-specific insertion of a coding sequence to direct the expression of heterologous proteins to the mammary gland of lactating females (Cre-mediated knockin mutants). Nine correctly targeted ES cell clones were identified by Southern blot using *Eco*R1 restriction digest in combination with a 3-prime probe that was not part of the targeting vector. Although more than 8 kb of homology sequence was used to construct the targeting vector, only about 5% of the neo^{pos}/tk^{neg} clones were correctly targeted. This might be the result of using non-isogenic DNA or the fact that the *Wap* locus is proximal to the centromere of chromosome 11.

Four ES cell clones (#59, #65, #69, and #107) were expanded and used for the production of 27 highly chimeric mice. Three ES cell lines (#59, #65, and #107) were transmitted though the germline of chimeric males. Subsequently, we bred the null allele of all three substrains into homozygosity (Fig. 1B,C). The mating of heterozygous mice resulted in homozygous mutants according to the expected Mendelian ratio of 25%, suggesting that Wap deficiency did not result in embryonic

lethality. Homozygous mutants developed normally until adulthood, and neither males nor females exhibited phenotypic abnormalities by 12–14 months of age.

Normal Mammary Gland Development in Wap-Deficient Females

The expression of the endogenous *whey acidic protein* locus is principally restricted to the mammary gland. During the course of mammary development from the virgin to the fully lactating female the steady-state levels of the *Wap* mRNA increases about 10,000-fold, with the most pronounced increase occurring around mid-pregnancy (Hennighausen *et al.*, 1991). Wap-deficient mice were fertile and exhibited a normal mating behavior. Females gave birth to normal litter sizes comparable to their wildtype controls ($Wap^{+/+}$ 6.9 ± 2.9; $Wap^{+/-}$ 7.2 ± 3.1; $Wap^{-/-}$ 6.8 ± 2.8, n = 162). Postpartum dams produced milk that was clearly visible in the abdominal region of the pups (data not shown). A histological examination of the mammary glands at day 1 of lactation revealed no phenotypic abnormalities between the knockouts and their age-matched lactating controls (Fig. 2A, upper panel). The whey acidic protein, which was abundant in secretory alveoli of lactating wildtype controls, was completely absent in Wap knockout dams as determined by immunohistochemistry (Fig. 2A, lower panel). As expected, the prolactin signaling cascade though Stat5a, which is imperative for the transcriptional activation of *Wap*, was not impaired in the mutants, as demonstrated by nuclear localization and phosphorylation of Stat5 (Fig. 2B, upper panels). Wap-deficient mammary epithelia exhibited a typical expression of ductal (Nkcc1) and alveolar (Npt2b) cell type-specific markers in virgin and lactating females (Fig. 2B, lower panels), suggesting that the lack of the whey acidic protein does not affect cell specification and functional differentiation.

We further examined the transcriptional activation of β-*casein* (*Csnb*), α-*lactalbumin* (*Lalba*), *Wdnm1*, and *Wap* mRNA in two wildtype females as well as three heterozygous and three homozygous knockouts at day 1 of lactation (Fig. 3A). Each heterozygous and homozygous mutant represents a pair from a substrain derived from the three targeted ES cell lines that were passed through the germline of chimeric males (lines #59, #65, and #107 in that order). The northern blot analysis demonstrated that the targeting strategy led to a complete transcriptional repression of the *Wap* locus. Furthermore, Wap deficiency had no effect on β-*casein* or α-*lactalbumin* mRNA expression. Like Wap, Wdnm1 is a member of the four-disulfide-core-domain (4-DSCD) protein family that is developmentally regulated in the mammary gland (Simpson *et al.*, 2002). The expression of *Wdnm1*, however, is not linked to terminal differentiation and precedes the expression of *Wap*. The *Wdnm1* mRNA is not upregulated in Wap-deficient mammary epithelia. In addition, we did not observe significant differences in *Wap* mRNA expression between heterozygous mutants and wildtype controls, which indicated that *Wap* haploinsufficiency resulted in the upregulation of the remaining wildtype allele. The absence of the whey acidic protein in mammary gland tissues from lactating females of the three Wap knockout substrains was verified by western blot analysis (Fig. 3B). In analogy to the northern blot results, heterozygous Wap knockout females exhibited levels of the whey acidic protein that were equivalent to wildtype controls. In addition, we examined the amount of caseins and whey proteins in the milk of nursing females around day 15 of lactation using SDS-polyacrylamide gel electrophoresis and Coomassie blue staining (Fig. 3C). Although the whey

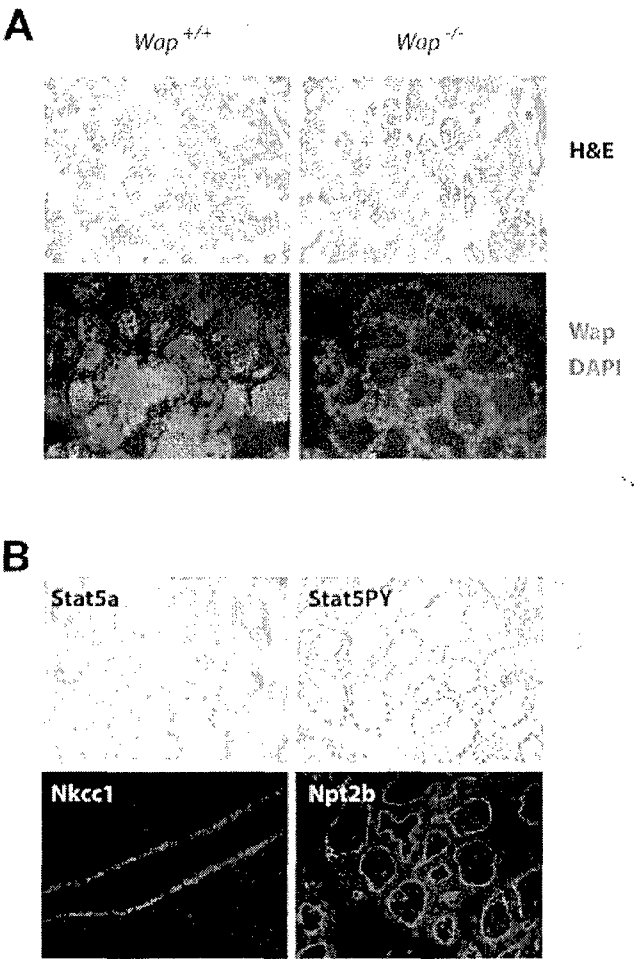


FIG. 2. Mammogenesis in Wap-deficient females (−/−) and their wildtype (+/+) controls. **A:** Upper panel: histological examination of mammary tissue at day 1 of lactation (hematoxylin and eosin staining, magnification 40×); lower panel: immunostaining of the whey acidic protein (Wap) in secretory alveoli of the mammary gland at day 1 of lactation (magnification 200×). **B:** Upper panel: Examination of Stat5a and Stat5-pY nuclear localization in Wap-deficient dams at day 1 of lactation (magnification 200×); lower panel: immunostaining of ductal (Nkcc1) and alveolar (Npt2b) differentiation markers in nonpregnant (left) and lactating (right) $Wap^{-/-}$ females (magnification 200×).

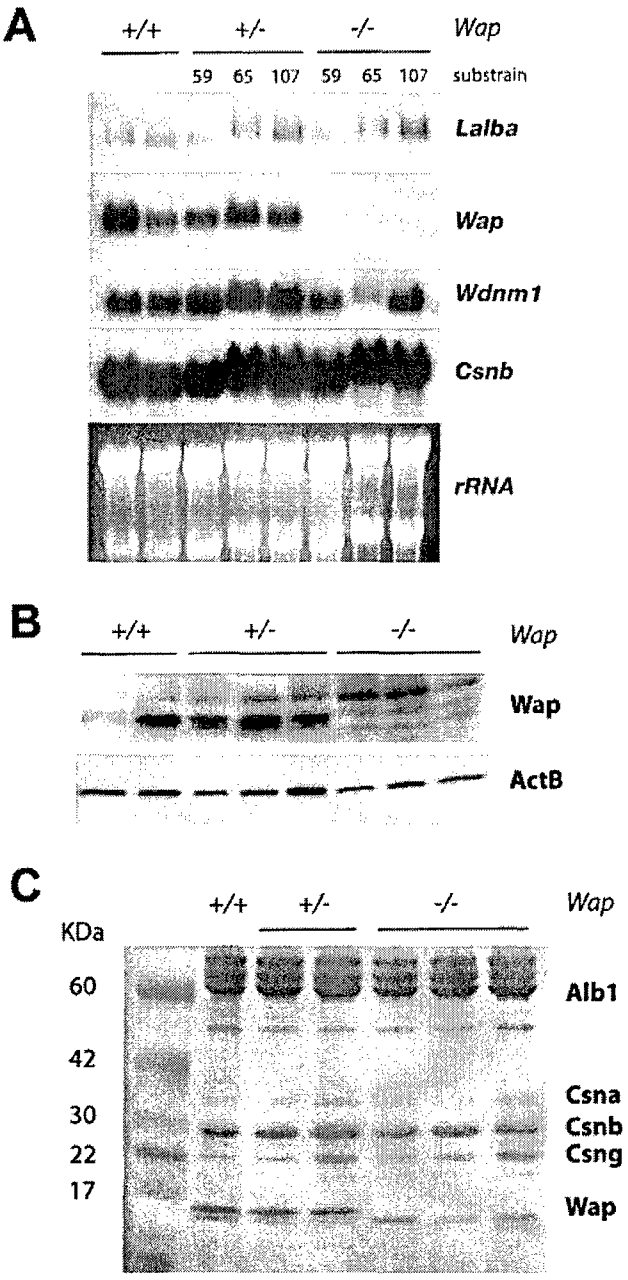


FIG. 3. Expression of major milk protein genes in the mammary gland (**A,B**) and milk (**C**) of Wap-deficient females (−/−) as well as heterozygous (+/−) and homozygous wildtype (+/+) controls at day 1 (**A,B**) and day 15 (**C**) of lactation. **A:** Northern blot analysis of β-*casein (Csnb), Wdnm1*, the *whey acidic protein (Wap)*, and α-*lactalbumin (Lalba)* mRNA. The 18S and 28S ribosomal RNA serves as a loading control. **B:** Western blot analysis to verify the absence of the whey acidic protein in the same samples shown in **A**. Detection of the β-actin (ActB) protein serves as a loading control. **C:** SDS-polyacrylamide gel electrophoresis and Coomassie blue staining of caseins and whey proteins in the milk of nursing females at day 15 of lactation.

acidic protein was clearly absent in the milk from knockout females, we did not detect any other significant variations in the amount of major milk protein fractions, in particular the caseins. This suggests that whey acidic protein is not essential for casein synthesis or the stability of proteins in the milk.

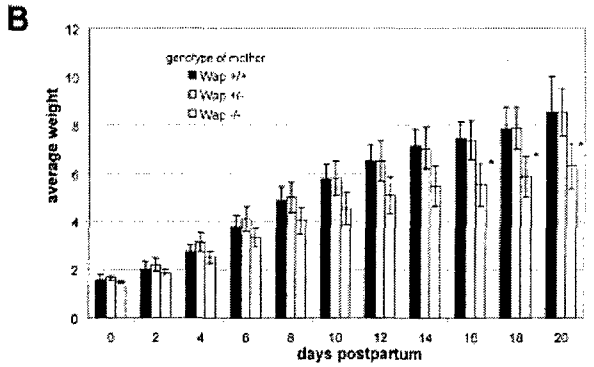
Presence of Wap in Milk Is Important for Adequate Nourishment of Offspring During the Second Half of Lactation

As shown above, Wap-deficiency had no effect on the terminal differentiation of secretory mammary epithelial cells. Nevertheless, we noted that many litters thrived poorly on $Wap^{-/-}$ dams, resulting in either lethality of pups or reduced body weight (Fig. 4A), in particular during the second half of the lactation period. This resulted in an unusually long lactation period of many WAP-deficient females, and their offspring could not be weaned before 4 or even 5 weeks of age. In some cases, pups of the substrain #59 nursed for 6 weeks before they could be separated from their mothers. Litters from control mice were weaned after a lactation period of 21–25 days. To confirm these subjective observations, we closely monitored the growth rate of the offspring from more than 50 lactating females of all genotypes in all three substrains (>500 pups) during their first and second lactation cycle (Fig. 4B,C). Entire litters were weighed at birth and every second day within the first 20 days of lactation. In consideration of the effects of small litter sizes on the average weight gain of the young, we excluded from this study lactating dams with fewer than six pups. The average birthweights of the pups were statistically indistinguishable between the genotypes of the mothers regardless of the genetic makeup of the offspring (i.e., whether the dams were mated with wildtype, heterozygous, or homozygous knockout males). This suggested that any intrauterine effects of Wap-deficiency were negligible. Beginning at day 4 of lactation, the pups suckling on Wap-deficient dams exhibited a growth disadvantage, which was statistically significant during the second half of lactation. The inclusion of the second lactation cycle into the analysis made the statistical differences more evident due to a greater number of measurements (Fig. 4C). It was previously reported that, in a number of genetically engineered strains, the extent of an abnormal mammary phenotype is more prominent during the first as compared to subsequent lactation periods (Liu *et al.*, 1998; Ormandy *et al.*, 1997). Our results, therefore, suggested that the abnormal phenotype caused by Wap deficiency is not reversible during the second lactation cycle.

Next, we examined whether the genotype of the young had any influence on the postnatal growth retardation. For this purpose, we mated Wap heterozygous knockout females with heterozygous males ($Wap^{+/-} \times Wap^{+/-}$). One hundred forty-nine mice from the resulting litters were weighed and genotyped at the age of 4 weeks (Fig. 4D). Since gender influences weight gain, we compared males and females separately. We did not observe statistically significant differences in the average weight among the various genotypes that were all nursed by Wap heterozygous knockout dams. As men-

D

genotype of pups	average weight of pups	
	Male	Female
Wap +/+	15.8 ±1.9 (n=13)	14.5 ±1.2 (n=27)
Wap +/-	14.8 ±0.9 (n=39)	13.1 ±1.5 (n=37)
Wap -/-	17.0 ±1.3 (n=18)	15.0 ±2.2 (n=15)



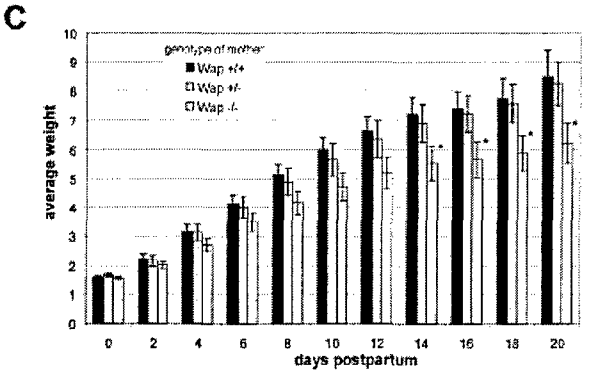


FIG. 4. Wap deficiency and growth rate of the nursing young. **A:** Comparison of the size of a malnourished pup suckling on a $Wap^{-/-}$ dam (left) and a normal weanling nursed on a $Wap^{+/-}$ heterozygous control dam (right) at day 21 of lactation. **B,C:** Average weight of pups during the first 20 days of lactation grouped by the genotype of the lactating mother and the first (**B**) or the first and second (**C**) lactation cycle of the dam. Error bars represent the confidence interval ($\alpha = 0.05$). **D:** Average weight of the offspring resulting from Wap heterozygous knockout breedings ($Wap^{+/-} \times Wap^{+/-}$) grouped by gender and genotype. Litters were weighed and genotyped at the age of 4 weeks.

tioned above, many pups nursing on $Wap^{-/-}$ dams were significantly smaller and could not be weaned by 4 or even 5 weeks of age. In summary, the combined studies suggested that the retarded development of the young is solely caused by the ablation of the whey acidic protein in the mammary gland and milk of $Wap^{-/-}$ lactating dams.

Wap Is Redundant as a Protease Inhibitor in the Milk

Many 4-DSC domain proteins (i.e., those carrying the "WAP motif") are suggested to function as protease inhibitors in various tissues. The analysis of the major protein fractions in the milk of Wap knockout mice and their controls (Fig. 3C) suggested that the founding member of this protein family is not essential for the inhibition of proteases. Wap deficiency did not alter the stability and equal quantity of caseins in the milk. To validate this observation, we performed a colorimetric assay to measure the endogenous protease activity in milk from Wap-deficient dams and their wildtype controls at day 15 of lactation (Fig. 5A). The protease assay was carried out at two time points: 1) immediately after retrieving the milk (day 1), and 2) after storing the milk overnight in a refrigerator (day 2). As expected, refrigerated milk exhibited a slightly higher endogenous protease activity in both the knockout and wildtype controls. At both time points, milk deficient in the whey acidic protein exhibited a marginally elevated protease activity. These differences, however, were statistically insignificant. Next, we examined whether Wap deficiency changes the capability of milk to buffer increasing amounts of exogenous proteases (Fig. 5B). This is an important characteristic of milk, which influences its digestibility in the stomach and gut of the suckling offspring. In comparison to the standard curve (reaction buffer control, no milk), milk samples from Wap-deficient dams and their controls were able to buffer low concentrations of exogenous trypsin in a very similar manner in our experimental setting, and the differences between both study groups were not statistically significant. Collectively, our results suggested that the whey acidic protein is not an essential protease inhibitor, and its absence from milk does not alter the stability of major milk proteins, the activity of endogenous proteases, or the ability of milk to buffer low concentrations of an exogenous protease in a significant manner.

Wap Deficiency in the Milk Does Not Affect the Glutathione (GSH) Antioxidant System in the Offspring

Whey protein concentrates serve as nutritional supplements during times of high physical activity, stress, and illness. Whey proteins are rich in cysteine, which is a crucial limiting amino acid for intracellular glutathione (GSH) synthesis. The GSH antioxidant system is the principal protective mechanism of the cell, and it is an essential factor in the development of an immune response. Therefore, whey proteins serve as effective and safe cysteine donors for GSH replenishment during GSH depletion in immune compromised states. Due to their positive effects on the immune system, whey proteins have also been suggested to possess anticancer activities (Bounous *et al.*, 1991). Since pups, regardless of their genetic backgrounds, thrive poorly on Wap knockout dams, we hypothesized that Wap deficiency in the milk might affect GSH levels in the offspring, resulting in poor general health. To address this issue, we measured GSH levels in the spleen and thymus of four severely malnourished pups from Wap-deficient dams and four pups of

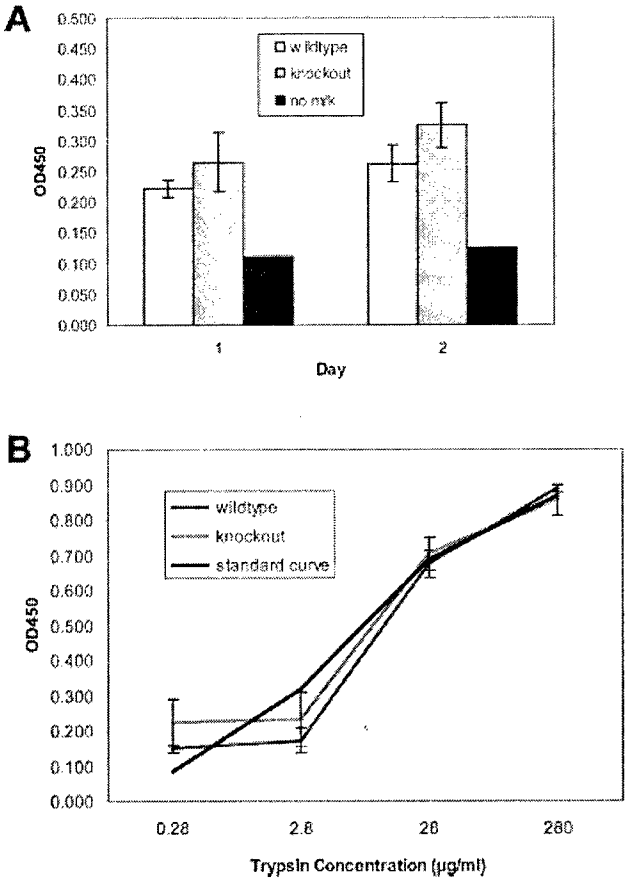


FIG. 5. Colorimetric protease assay. **A:** Determination of the endogenous protease activity in milk from Wap-deficient dams and their wildtype controls at day 15 of lactation. The assay was carried out as described in Materials and Methods immediately after retrieving the milk (day 1) and after storing the milk overnight in a refrigerator (day 2). The background absorbance of the colorimetric assay was established by a "no milk" (reaction buffer only) control. **B:** Mean protease activity of trypsin in the milk from Wap knockout females and their wildtype controls. Note that although milk lacking Wap has a slightly higher endogenous protease activity compared to milk from wildtype controls ($P > 0.05$), both milk types are equally able to buffer trypsin at a low concentration in comparison to the "no milk" (reaction buffer only) standard curve. Error bars in **A** and **B** represent confidence intervals ($\alpha = 0.05$).

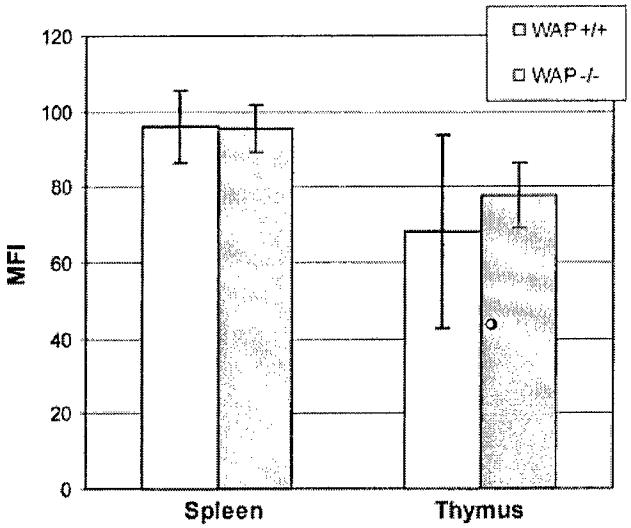


FIG. 6. Glutathione levels in splenocytes and thymocytes derived from four 15-day-old severely malnourished pups suckling on Wap-deficient dams ($Wap^{-/-}$) and from four average-size pups nursing on wildtype control females ($Wap^{+/+}$). Glutathione levels within individual cells were measured as fluorescence by flow cytometry. The mean fluorescent intensity (MFI) represents at least 10^4 individual measurement points per animal and tissue type. Error bars represent the confidence intervals ($\alpha = 0.05$) between animals grouped by tissue type and genotype of the lactating mother.

average size suckling on wildtype control females at day 15 of lactation. Using a flow cytometry-based glutathione assay (Roederer *et al.*, 1991), we were unable to detect statistically significant differences in the amount of intracellular GSH levels in either splenocytes or thymocytes (Fig. 6). Hence, while whey acidic protein in the milk is required for adequate nourishment of the offspring, it is not a major nutritional source for cysteine to establish and maintain the GSH antioxidant system in the offspring.

DISCUSSION

The mammary gland-specific expression of the *Wap* gene increases more than 1,000-fold from the virgin state to late pregnancy and lactation (Pittius *et al.*, 1988). It had been hypothesized that Wap is a protease inhibitor, and it may have a role in remodeling the mammary gland at specific stages of the lactation cycle (Simpson *et al.*, 2002). The generation and analysis of mice lacking *Wap*, however, reveal that this milk protein gene is not required for functional differentiation. This is a surprising observation, since the expression of this gene is frequently applied as an advanced differentiation marker in a variety of in vivo and 3D cell culture model systems. Clearly, Wap is not required to suppress alveolar proliferation during functional differentiation, as suggested from various transgenic studies in mice and pigs (Burdon *et al.*, 1991; Hennighausen *et al.*, 1994; Nukumi *et al.*, 2004; Shamay *et al.*, 1992). In addition, we found that Wap is dispensable as a protease inhibitor to maintain the stability of abundant secretory proteins in the milk as anticipated from its 3D structure and suggested functions of other Wap-like 4-DSC domain proteins in different tissues. Our findings are in compliance with unpublished studies by Simpson and Nicholas (University of Melbourne, Australia) that show that HPLC-purified whey acidic protein from the tammar wallaby did not exhibit evidence of inhibition of either trypsin or chymotrypsin in a colorimetric assay that uses a 10-fold excess of Wap over the two proteases (Kaylene Simpson, pers. commun.). Moreover, Simpson and Nicholas discussed that all four disulfide bridges are required for a protease activity, and they questioned whether domain I of the mouse whey acidic protein is functionally active due to the absence of cysteine residues 1 and 8 to complete the

folding unit (Simpson *et al.*, 2002). While Wap might not act as a general protease inhibitor, the authors hypothesized that this protein might function as a specific inhibitor of unidentified milk proteases. Our observations in Wap knockout mice do not exclude this possibility, but it is evident that other, less abundant protease inhibitors are able to compensate for the loss of Wap in the milk.

Despite normal mammogenesis, a significant number of litters thrived poorly on Wap-deficient dams, in particular during the second half of lactation. This phenotype was intrinsic to the genotype of the lactating dam but not to the nursing offspring. We also excluded the possibility that Wap deficiency affects the immune system of the young through reduced amounts of cysteine in the milk and subsequent glutathione deprivation. The growth retardation observed during the second half of pregnancy might be caused by a reduced milk protein supply that does not fully meet the needs of the growing litter, which triples in size within the first 10 days of lactation. Thus far, we have not experimentally addressed whether Wap is required for the maturation of the digestive system of the offspring. While extracting milk from the mammary glands at day 15 of lactation for the protease assays, we also noticed that the mammary glands of a subset of Wap knockout dams contained less milk compared to their wildtype controls. The skin of 1-week-old malnourished pups was dry, which is another indication of a lack of fluids. A histological analysis of these glands revealed that, although well-differentiated secretory acini were present in all specimens, individual alveoli appeared to be smaller (data not shown). The histological appearance, however, varied quite considerably between knockout animals, and a number of Wap-deficient mammary glands were indistinguishable from their wildtype controls. We therefore hypothesize that another modifier locus might be responsible for this phenotypic variation among knockouts at the histological level since all studies were performed in a C57Bl6/129Svev mixed genetic background. To address this issue, we are currently backcrossing the Wap knockout allele into various inbred strains, which is the basis for mapping loci that modify this phenotypic variation.

The surprising finding that Wap is not required for terminal differentiation has implications for the biology of pluripotent mammary progenitor cells. Using a *Wap-Cre/Rosa-LacZ* double transgenic reporter system, we recently discovered parity-induced mammary epithelial cells (PI-MECs), which are abundant in nonpregnant, parous females and are virtually absent in nulliparous (virgin) animals (Wagner *et al.*, 2002). In synchrony with the endogenous locus, the *Wap* gene promoter-driven Cre recombinase transgene specifically targets hormone-responsive alveolar cells undergoing an advanced differentiation program during the second half of pregnancy and during lactation. Through the Cre-mediated excision of a transcriptional *Stop* sequence between the promoter and the *LacZ* gene, the transient upregulation of Cre recombinase permanently activates a ubiquitously expressed *Rosa-LacZ* reporter transgene (Soriano, 1999), whose expression (unlike the *Wap-Cre* construct) is not dependent on the differentiation status of a given cell. Hence, the constitutive activation of the reporter transgene labels differentiating cells during pregnancy and lactation and their descendents (i.e., cells that are apoptosis-resistant and that silence the *Wap* locus during mammary gland remodeling following a normal pregnancy-lactation cycle). Thus, the LacZ-expressing cells in the remodeled gland represent an epithelial subtype (PI-MECs), which is not present in the virgin gland. Unexpectedly, PI-MECs exhibited various features of multipotent mammary epithelial stem cells. Upon transplantation into an epithelial-deprived mammary gland fat pad, these LacZ-expressing epithelial progenitors are able to self-renew and contribute to ductal and alveolar morphogenesis in the reconstituted mammary gland (Wagner *et al.*, 2002). Our observation suggested that a subset of hormone-responsive cells expressing advanced differentiation markers (i.e., *Wap*) maintains or regains characteristics of stem cells. This potentially paradigm-shifting observation has been scrutinized in the peer-review process at a study section at the NIH Center for Scientific Review. The critique mainly focuses on the hypothetical limitation of the labeling methodology that a Cre-lox-based cell fate-mapping technique cannot discriminate cells with high or low levels of transient *Wap-Cre* expression. This critique is, therefore, centered on the commonly accepted paradigm that the presence of the whey acidic protein and, more importantly, the level of *Wap* expression determine the advanced or "terminal" differentiation status of a given cell. To test whether this critique is legitimate was another strong motivator to generate the Wap knockout model. The results of this study clearly demonstrate that *Wap* is not required for terminal differentiation. In conclusion, the upregulation of *Wap* might be a valid indicator for an advanced differentiation profile of the *entire* mammary gland. A difference in *Wap* expression between individual cells, however, may not serve as an indicator for the terminal differentiation status and the fate of a cell during involution (i.e., whether a cell will live or die). Therefore, the Cre-lox-based cell fate-mapping technique is, indeed, a valid method to label differentiating, pregnancy hormone-responsive cells that activate *Wap* regulatory elements.

MATERIALS AND METHODS

Construction of the *Wap* Targeting Vector

BAC clone encompassing the *Wap* locus was isolated from a mouse 129SvJ genomic library (Incyte Genomics). Contiguous *Kpn*1 and *Kpn*1/*Eco*R1 fragments harboring 4.5 kb of the promoter, the entire *Wap* gene, and more than 1.8 kb of the 3′ sequence were subcloned into pZErO (Invitrogen). Sequencing all exons and introns as well as part of the 3′ flanking DNA was pivotal for determining the targeting strategy and the design of the 3′ outside probe. A 3.7-kb fragment containing exons 2 to 4 and part of the 3′ sequence was

amplified using Pfx polymerase (5′-CCG CTC GAG CGA ATT CTC ACC TTA CTA CCG GGT GTG-3′; 5′-CCG CTC GAG GCG GCC GCG AGT GGA TGG AAC CTT AAT TGA AG-3′), introducing an *EcoR*1 site 5′, a *Not*1 site 3′, and two *Xho*I sites on either end of the amplification product. The PCR product was cut with *Xho*I, cloned sticky into the *Xho*I site of pZErO, and sequenced. The 3′ targeting arm was released by *Xho*I/*Not*1 digest and directionally cloned into the *Xho*I and *Not*1 sites downstream of the floxed neomycin cassette of pLoxpNeo (Xu *et al.*, 1999). The targeting vector was completed by subcloning a 4.5 kb *Kpn*1 fragment, containing the promoter and the 5′ UTR of *Wap,* into the *Kpn*1 site upstream of the floxed neomycin cassette. The correct orientation of the 5′ arm was verified by PCR (5′-TAG AGC TGT GCC AGC CTC TTC-3′; 5′-GAT CTA TAG ATC TCT CGT GGG ATC-3′), and all cloning junctions of the final targeting vector were sequenced. The targeting vector was linearized using *Not*I, phenol-chloroform extracted, and electroporated into R1 cells (20 μg DNA per 10^7 ES cells). The selection and expansion of ES cell clones were performed at the Germline Mutation Core Facility (GMCF) at NCI.

Southern Blot Analysis

Genomic DNA from 178 ES cell clones was prepared using standard phenol/chloroform extraction. Fifteen μg of DNA was digested with *Eco*RI at 37°C overnight and separated on a 0.8% agarose gel. The DNA was denatured and blotted onto a nylon membrane (GeneScreen plus, NEN, Boston, MA), and hybridized overnight with a ^{32}P-labeled probe at 65°C using QuickHyb (Stratagene, La Jolla, CA). The 3′ external probe, ~550 bp in size, was generated by PCR (5′-CTT GAA GCC TTA GCT AAC GTG G-3′ and 5′-TGG GTT CTC CCA CAC CAA TGA C-3′). Membranes were washed in 0.1× SSC buffer containing SDS and exposed for 16–24 h to a Kodak XOMAT-AR film. The *Eco*R1 Southern analysis yielded two distinct bands of 6.7 kb (wildtype allele) and 4.6 kb in size (knockout allele).

Generation of Wap Knockout Mice and Genotyping Protocols

Four correctly targeted ES cell clones (#59, #65, #69, and #107) were used for the production of chimeric mice. The injection of ES cells into C57/Bl6 blastocytes was carried out at the Germline Mutation Core Facility (GMCF). The germline transmission of the *Wap* knockout allele [*Wap*$^-$ or Wap^{tm1Kuw}] was verified using the Southern blot assays described above. A single PCR assay using three primers (132 5′-TAG AGC TGT GCC AGC CTC TTC-3′; 136 5′-GTT CTC CAA GCC ACA CCC GG-3′; and 1823 5′-GTG CTG TCC ATC TGC ACG AGA C-3′) was developed to genotype mice that carry one or two *Wap* knockout alleles. Amplicons for the wildtype (230 bp) and null allele (330 bp) were clearly distinguishable on a 2% agarose gel. All animals used in the studies were treated humanely and in accordance with federal guidelines and institutional policies.

Histology and Immunohistochemistry

The fourth inguinal mammary glands of Wap knockout mice and their controls were resected, fixed overnight at 4°C in 10% buffered formalin (Fisher Scientific, Pittsburgh, PA), dehydrated, paraffin-embedded, and sectioned. Sections were rehydrated and stained with hematoxylin and eosin (H&E, Vector Laboratories, Burlingame, CA) for general histology. The immunohistological detection of the whey acidic protein, Nkcc1, Npt2b, Stat5a, and Stat5a/b (Tyr694/Tyr699) was performed as described previously (Nevalainen *et al.*, 2002; Shillingford *et al.*, 2002; Wagner *et al.*, 1997). An AlexaFluor 488-conjugated secondary antibody (Molecular Probes, Eugene, OR; Invitrogen) was used to visualize Wap, Nkcc1, and Npt2b. Slides were counterstained with DAPI, which was a component of the Vectashield mounting medium (Vector). Due to low immunofluorescence of the Stat5 proteins, we used biotinylated secondary antibodies to detect Stat5a and the phosphorylated form of Stat5a/b instead. Vectastain Elite ABC and DAB peroxidase substrate kits (Vector) were used to complete the color reaction. Slides were counterstained briefly with hematoxylin to visualize Stat5 negative nuclei. Brightfield and fluorescence images of histological slides were taken on a Nikon Labophot microscope equipped with a Nikon Coolpix 990 camera as well as FITC and DAPI filter sets.

Northern Blot Analysis

The isolation of total RNA from mammary tissue has been described previously (Wagner *et al.*, 1997). Twenty μg of total RNA was separated on a 1.5% formaldehyde gel and transferred to a GeneScreen Plus membrane. Transcripts of milk protein genes were detected by probing the membranes with ^{32}P end-labeled antisense oligonucleotides (*Wap* 5′-CAA CGC ATG GTA CCG GTG TCA-3′; *Csnb* 5′-GTC TCT CTT GCA AGA GCA AGG GCC-3′) or ^{32}P randomly labeled cDNA probes (*Wdnm1* and *Lalba*). The α-*lactalbumin* cDNA was a kind gift from Gertraud Wasner Robinson and Lothar Hennighausen (NIH). The cDNA probe for *Wdnm1* was cloned by RT-PCR from total RNA of lactating mammary tissue using an oligo $dT_{12\text{-}18}$ primer for reverse transcription and the following *Wdnm1*-specific primer pair for the PCR amplification: 5′-GTC AGA GCC AAC ATG AAG AC-3′; 5′-GGA TGC TAA GGA TAG TTT ATT TTA G-3′. The hybridization was performed overnight at 55°C (oligo probes) or 65°C (cDNA probes) using QuickHyb (Stratagene). Membranes were washed in 1× SSC buffer containing 0.1% SDS and exposed for 6–12 h to Kodak XOMAT-AR film.

Milk Protease Assay

Pups were removed from their lactating mothers several hours before both #4 inguinal mammary glands were retrieved. Milk was separated by slow-speed centri-

fugation through a cell strainer into a 50 ml Falcon tube. We normally obtained 100–500 µl milk from both controls and knockout dams; only a subset of lactating Wap-deficient dams had less than 100 µl. The milk was transferred into a 1.5-ml centrifuge tube and refrigerated. A small portion of the milk that contained red blood cells on the bottom of the Falcon tube was discarded. To measure the endogenous protease activity of milk, we used the colorimetric Protease Determine Quick Test (PDQ) assay from Athena Enzyme Systems Group (Baltimore, MD). This assay measures the activity of a wide range of proteases described in the manufacturer's protocol. In brief, the storage solution was removed from the PDQ vials after maintaining those for an hour at room temperature. Next, we added either 0.5 ml of the pH 8.0 Tris buffer (negative control) or our test samples containing 50 µl milk in 0.45 ml Tris buffer. Vials were incubated for 1, 2, or 3 h at 37°C. To stop the reaction and to amplify color, 1.5 ml of a 0.1 N NaOH solution was added to each vial. The liquid content was transferred to standard cuvettes to record the absorbance spectrophotometrically at 450 nm. Additionally, we prepared a 10-fold dilution series of trypsin (e.g., 280–0.28 µg/ml) in the supplied Tris buffer to construct a standard curve. The optical density at 450 nm is directly proportional to the enzyme activity. To determine the capacity of milk to buffer the activity of trypsin, we added 50 µl of milk into 0.45 ml of Tris buffer, which contained various dilutions of the protease in accordance with the standard curve.

Glutathione Assay

Splenocytes and thymocytes were derived from four 15-day-old, severely malnourished pups suckling on Wap-deficient dams and from four, average-size pups nursing on wildtype control females. Glutathione (GSH) levels within individual cells were measured using a flow cytometry-based assay (Roederer *et al.*, 1991). The mean fluorescent intensity (MFI) represents at least 10^4 measurement points per animal and tissue type.

Statistical Analyses

Mean values shown in Figures 4–6 are accompanied by a confidence interval (CI). Nonoverlapping CIs represent significant differences at a significance level of 0.05. The statistical significance was verified or tested for those groups with overlapping CIs using the *t*-test or nonparametric Mann-Whitney (U) test.

ACKNOWLEDGMENTS

The authors thank Drs. Lothar Hennighausen and Gertraud Robinson (LGP, NIDDK) for providing the α-*lactalbumin* cDNA as well as the anti-Stat5 and anti-Wap antibodies. We also thank Dr. Andrea Krempler (University of the Saarland, Germany) for mapping the BAC clone and subcloning *Wap* gene sequences while she was a postdoctoral fellow in K.U.W.'s laboratory. The anti-Nkcc1 and anti-Npt2b antibodies were a kind gift from Dr. Jim Turner (NIH) and Dr. Jurg Biber (University of Zurich, Switzerland). The authors also thank Dr. Simpson and Dr. Nicholas (University of Melbourne, Australia) for sharing their unpublished observations on the role of Wap as a protease inhibitor.

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Loss of *Tip30* rapidly immortalizes murine mammary epithelial cells and leads to ductal hyperplasia in the mammary gland

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Key Words: Tip30, Mammary Gland, Hyperplasia, Tumorigenesis

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Abstract

We show that deletion of the *Tip30* gene, encoding a repressor for estrogen receptor alpha, leads to ductal hyperplasia in mouse mammary glands early in life and extensive mammary hyperplasia and periductal lymphocyte infiltration with age. *Tip30*$^{-/-}$ mammary glands, when transplanted into wild-type mammary fat pads, also display mammary trees with ductal hyperplasia. Tip30 loss promotes proliferation of primary mammary epithelial cells (MECs) and can cooperate with the oncogene, *H-RasV12*, to increase cell growth. Strikingly, loss of Tip30 results in rapid immortalization of MECs in vitro and a significant decrease in E-cadherin expression. Therefore, our results suggest that Tip30 is a negative regulator of mammary epithelial cell proliferation, and its absence in the mammary gland may predispose them to tumorigenesis.

Significance

Transformation of MECs from the normal to the preneoplastic state before becoming fully neoplastic requires alteration of expression of certain genes in these cells. Our work provides the first evidence demonstrating that Tip30 is a key negative regulator of proliferation of MECs using a genetically-engineered mouse model lacking Tip30. A single step, deleting the *Tip30* gene, leads to a rapid immortalization of murine primary MECs, suggesting that Tip30 may play an important role in the suppression of mammary tumorigenesis. Thus, human Tip30 may represent a new valuable diagnostic and therapeutic target for breast cancer.

Introduction

In the mammary gland, as in other organs, the progression of a cell from normal to neoplastic is a multi-step process. Experimental evidence suggests that mouse mammary epithelial cells advance through a period of hyperplasia before becoming neoplastic (Medina 1993; Medina 2002). A number of mouse models support this finding, with the appearance of mammary epithelial cell hyperplasias occurring early in life, and mammary tumors arising stochastically after a long latency. Balb/c mice heterozygous for p53 develop stochastic mammary carcinomas after a relatively long latency of 8 to 14 months. Mammary epithelial hyperplasia is detected in all of these mice regardless of whether they develop palpable lesions. Mice homozygous for the p53 gene deletion did not develop mammary tumors at a high incidence. The mammary glands of the majority of these $p53^{-/-}$ mice do, however, display preneoplastic changes at an early age, such as epithelial hyperplasia (Kuperwasser 2000). Mammary glands of Brca1 conditional mutant mice display hyperplastic areas by 2 to 10 months of age, but no tumors. After a latency of 10-13 months, spontaneous mammary tumors do develop (Xu 1999). In several transgenic mice, such as doxycycline-inducible human c-*myc* or MMTV-cyclin D1 models, mammary hyperplasia first appears by 30 days to 2 months and increases in severity as the mice age. Mammary tumors will appear in those mice after a long latency (Wang 1994; Cruz 2001). Although several mouse models display hyperplasia of the mammary glands appearing early in life with the eventual appearance of tumors after a long latency, other mouse models, such as the cdc25B transgenic and the P-cadherin and Caveolin-1 knockout models, exhibit mammary epithelial hyperplasia that does not progress to mammary tumor formation (Radice 1997; Ma 1999; Lee 2002). In all of these models, the development of hyperplasia before the appearance of solitary mammary tumors suggests that additional changes are required for tumor development. Acquisition of additional genetic changes may,

therefore, lead to mammary tumor formation. Thus, identification of genes that are altered in preneoplasia will not only help us understand the molecular basis of tumorigenesis but also provide diagnostic and therapeutic targets.

Tip30 is a tumor suppressor which can promote apoptosis and inhibit angiogenesis (Shtivelman 1997; Xiao 1998). Its expression is altered in human prostate, lung, colon, and breast cancers (Hewitt 2000; Varaambally 2002; Lee 2004). We previously reported that somatic mutations in the *Tip30 gene* were found in HCC patients (Ito 2003). Tip30-deficient mice of C57BL6/J and 129SvJ mixed genetic background spontaneously develop tumors by 18-20 months of age (Ito 2003). The majority of these tumors occur in female mice, and many of them arise in estrogen-targeted organs (Ito 2003). This is consistent with the finding that Tip30 acts as a repressor in estrogen receptor alpha-mediated transcription in both estrogen-dependent and -independent manners (Jiang 2004), suggesting that Tip30 may play a role in the development and tumorigenesis of estrogen-targeted organs, such as the mammary glands.

Here we describe the effects of loss of *Tip30* on mammary gland development. *Tip30*-null mice develop mammary epithelial hyperplasia at an early age. By one year of age, these mice show an increased occurrence of alveolar buds in the mammary glands. MECs from *Tip30*-deficient mice proliferate at an increased rate *in vitro* as compared to wild-type cells. These *Tip30*-deficient MECs also give rise to immortal clones faster than wild-type MECs *in vitro*. These observations suggest that alteration of Tip30 may be an early lesion which contributes to the progression of mammary epithelial cells from the normal to the neoplastic state.

Results

Tip30 colocalizes with estrogen receptor alpha in mammary epithelial cells

Previous work in our lab has shown that Tip30 acts as a repressor of estrogen receptor alpha (ERα) mediated transcription and Tip30 loss increases expression of ERα-targeting gene *c-myc* in the mammary gland of virgin mice (Ito 2003; Jiang 2004). To further investigate the functional relationship between Tip30 and ERα, we sought to determine whether Tip30 and ERα colocalize in the nucleus of mammary epithelial cells in vivo. The same sections of mammary glands from 8-week-old Tip30 wild-type virgin mice were immunostained for both Tip30 and ERα. Confocal microscopy analysis showed significant co-localization of Tip30 with ERα in the nuclei of wild-type mammary epithelial cells (Fig. 1). We then demonstrated that the cellular localization of Tip30 is developmentally regulated in the mammary gland (see supplemental data). This indicates that Tip30 may exert its effects on proliferation of mammary epithelial cells and regulate mammary development and tumorigenesis.

Loss of Tip30 results in ductal hyperplasia in virgin mice early in life.

To investigate the role of Tip30 in mammary gland development and tumorigenesis, we analyzed the phenotypic consequences of Tip30 deficiency on mammogenesis in genetically engineered mice by examining whole mount stained mammary glands from 8-week-old virgin *Tip30*$^{+/+}$ and *Tip30*$^{-/-}$ mice. As shown in Fig. 2, mammary ducts of both the wild-type and *Tip30*$^{-/-}$ female mice penetrated the entire mammary fat pad, indicating that Tip30 is not essential for ductal elongation (Fig. 2a-b). However, most of the main ducts in *Tip30* mice were noticeably thicker than in the wild-type littermates. To investigate this more closely, we examined sections of the whole mount specimens. Figure 2 shows that *Tip30*$^{-/-}$ mammary glands had a normal composition of epithelial cells with an extended lumen but much enlarged luminal ducts (Fig. 2d), as compared to those of wild-type littermate controls (Fig. 2c). In

comparison to those of wild-type littermate controls (Fig. 2e, g), *Tip30*$^{-/-}$ ducts appeared to have a hyperplasia that is characterized by an increased layer of crowded ductal cells and cells focally forming micropapillary fronds (Fig. 2f, h). In addition, there is clear evidence of increased mitotic activity and individual necrotic epithelial cells characterized by nuclear karyorrhexis, cytoplasmic eosinophilic change and fragmentation, and association with occasional neutrophils. These results suggest that Tip30 loss results in ductal hyperplasia in mammary glands early in life.

Tip30-null mice have an increased incidence of alveolar bud formation in the mammary glands with age.

Given the hyperplasia we observed in 8-week-old virgin *Tip30*$^{-/-}$ mice, we next sought to determine the effects of loss of Tip30 on the mammary glands of older virgin mice. We examined mammary glands from *Tip30*$^{+/+}$, *Tip30*$^{+/-}$ and *Tip30*$^{-/-}$ mice of C57BL/6, 129SVJ or FVB/C57BL6 (25% FVB / 75% C57BL/6) mixed background in the diestrus stage of the estrous cycle. Whole mounts (Fig. 3a) and histological sections (Fig. 3c) of mammary glands from virgin one-year-old *Tip30* wild-type and homozygous mice in diestrus were scored for the presence of ductal branching and alveolar buds as defined by Cardiff, *et al.* (Cardiff 2000). For mammary glands of all genetic backgrounds scored, 60-67% of the *Tip30* mammary glands displayed a significantly increased number of alveolar buds as compared to wild-type mammary glands (Fig. 3b). This 60-67% of *Tip30* mice from all backgrounds scored also showed significantly fewer mammary structures in the ductal branching stage as compared to wild-type mice. These data suggest that the loss of Tip30 increases the incidence of alveolar bud formation in the mammary glands of older virgin mice. Histological sections of mammary glands from these mice showed an increased number of alveolar buds as compared to wild-type mammary glands and the presence of ductal hyperplasia (Fig. 3c). Infiltration of lymphocytes around the ducts and blood

vessels was also observed in the mammary glands of 1-year-old Tip30$^{-/-}$ mice, but not in the mammary glands of their wild-type littermates (Table 1). These data show that in addition to the ductal hyperplasia observed in 8-week-old Tip30 knockout mice, older virgin female Tip30 knockout mice have an increased incidence of alveolar budding and lymphocyte infiltration in the mammary glands.

Transplantation experiments demonstrate that Tip30's effects on the mammary gland are intrinsic.

To determine whether the observed effects of loss of Tip30 on the mammary glands are due to the absence of Tip30 in other organs, such as the ovaries or pituitary gland, small pieces of mammary glands from 10-week-old *Tip30*$^{+/+}$ and *Tip30*$^{-/-}$ mice were transplanted into contralateral cleared fat pads of 21-day-old *Tip30*$^{+/+}$ (Fig. 4a) and *Tip30*$^{-/-}$ (Fig. 4b) mice. Ten weeks after transplantation, the mammary glands were harvested and sectioned. The transplanted *Tip30*$^{-/-}$ mammary glands removed from wild-type and knockout recipients displayed ductal hyperplasia as compared to transplanted *Tip30*$^{+/+}$ mammary glands (Fig. 4a). Additional transplantation experiments were performed where the transplanted mammary glands were harvested one-year post-transplantation. As with the short-term transplants, *Tip30*$^{-/-}$ mammary glands removed from wild-type recipients displayed ductal hyperplasia as compared to transplanted wild-type mammary glands (data not shown). There was also an increase in alveolar budding in the transplanted *Tip30*$^{-/-}$ mammary glands as compared to the wild-type transplants (Fig. 4c). In addition, lymphocyte infiltration was observed near some blood vessels and ducts in the mammary glands of both the wild-type and knockout transplanted glands (Fig. 4d-e). The infiltrates occurred much more frequently in the transplanted knockout glands, however (Table 1). These results suggested that the effects of Tip30 deficiency on the mammary glands are intrinsic.

Loss of Tip30 increases mammary epithelial cell proliferation.

To assess Tip30's role in the proliferation of MECs we used *in vivo* BrdU labeling in 8-week-old virgin $Tip30^{+/+}$ and $Tip30^{-/-}$ mice on the C57BL/6 background (Fig. 5a). Many BrdU-labeled MECs (approximately 12%) were observed in the $Tip30^{-/-}$ mammary glands whereas few BrdU-labeled epithelial cells (approximately 1%) were observed in wild-type mice. We also determined that loss of Tip30 did not significantly increase apoptosis in the mammary gland by the in situ Tunel assay (data not shown). This result demonstrated that $Tip30^{-/-}$ MECs in mature virgin mice proliferate faster than $Tip30^{+/+}$ epithelial cells *in vivo*. To verify this, we next examined the effect of Tip30 loss on the growth of primary MECs *in vitro*. Primary MECs of virgin $Tip30^{-/-}$ and $Tip30^{+/+}$ mice from the same litters were isolated and confirmed by in situ immunostaining of E-cadherin (data not shown), and their growth rates were compared. As expected, we found that $Tip30^{-/-}$ cells grew much faster than $Tip30^{+/+}$ cells as demonstrated by both viable cell counting and MTT assays (Fig. 5b). This result suggests that loss of Tip30 promotes proliferation of MECs.

To test whether loss of Tip30 could cooperate with oncogenes to increase the proliferation of MECs, we infected early-passage $Tip30^{+/+}$ or $Tip30^{-/-}$ MECs with pBabepuro or pBabepuro-H-Ras-V12 retroviruses. After three days of selection with puromycin, MECs were seeded onto plates for viable cell counting and MTT assays. As shown in Figure 5c, H-Ras-V12 increased the growth rate of both *Tip30* wild-type and *Tip30*-null MECs. The $Tip30^{-/-}$ MECs infected with H-Ras-V12 grew at a much faster rate as compared to wild-type MECs infected with H-Ras-V12 or *Tip30*-null MECs infected with pBabepuro control retrovirus. The Tip30 wild-type MECs infected with H-Ras-V12 grew at a rate similar to *Tip30*-null MECs infected with pBabepuro, suggesting that $Tip30^{-/-}$ MECs are prone to

increased cell proliferation by overexpression of Ras-V12. Together, these data suggest that Tip30 plays an important role in the regulation of MEC proliferation in virgin mice.

Deletion of the *Tip30* gene, a single step, results in a rapid immortalization of mouse MECs.

Mouse primary MECs in culture undergo crisis at about passage 3 to 5, during which time they expand very slowly. After crisis, which can last several weeks, immortalized cells, which proliferate readily, will begin to appear (Medina 2000). While working with wild-type and *Tip30*-null MECs in culture, we noticed that the *Tip30*$^{-/-}$ cells continued to proliferate even after the wild-type cells had entered the crisis stage (Fig. 6a). To further analyze this, we constructed population doubling curves for *Tip30*$^{+/+}$ and *Tip30*$^{-/-}$ MECs. As shown in Figure 6b, proliferation of the wild-type MECs slowed dramatically after nine days in culture (i. e. seven population doublings). One week after this point they ceased proliferating altogether for four weeks. After 7 weeks in culture, immortal clones began to arise. However, the growth rate of these post-crisis wild-type MECs did not equal that of the pre-crisis MECs until another two weeks had passed. In contrast, *Tip30*-null MECs, while having a decrease in population doublings during the third week in culture (i.e. population doublings nine to ten), did not stop proliferating for any length of time. At two weeks post-crisis, the *Tip30*$^{-/-}$ MECs were proliferating at more than two times the rate of the pre-crisis *Tip30*$^{-/-}$ MECs. Significantly, the *Tip30*$^{-/-}$ MECs have divided over 340 times *in vitro*, whereas the *Tip30*$^{+/+}$ MECs have only undergone 120 population doublings. Immunoblot analysis of E-cadherin levels in the *Tip30*$^{+/+}$ and *Tip30*$^{-/-}$ MECs pre-crisis revealed that the *Tip30*$^{-/-}$ MECs had lower levels of E-cadherin as compared to the wild-type MECs (Fig. 6c). Post-crisis *Tip30*$^{-/-}$ MECs lost expression of E-cadherin and acquired a fibroblastoid morphology at a time when wild-type MECs had stopped proliferating, indicating that *Tip30*$^{-/-}$ MECs may have gone through epithelial mesenchymal transition. These data suggest that loss of Tip30 results in more rapid

immortalization of MECs in culture, and that *Tip30*$^{-/-}$ MECs maintain their growth advantage over wild-type MECs even after long periods in culture.

Discussions

Tip30 is a negative regulator of mammary epithelial cell proliferation

We demonstrate here that the loss of Tip30 in the mouse mammary gland leads to rapid immortalization of MECs *in vitro*, and ductal hyperplasia *in vivo*. We also show that *Tip30*$^{-/-}$ MECs proliferate faster than *Tip30*$^{+/+}$ MECs both *in vivo* and *in vitro* as demonstrated here by *in vivo* BrdU labeling, and *in vitro* viable cell counting and MTT assays. Loss of Tip30 cooperates with oncogenic Ras in promoting proliferation of MECs and may play a role in the epithelial mesenchymal transition. In addition, loss of Tip30 results in an increased incidence of alveolar budding and lymphocyte infiltration in the mammary glands of older virgin mice. The effects of Tip30 described here are due to the loss of Tip30 in the MECs themselves, as shown in transplantation experiments. Tip30, therefore, plays an important role in the regulation of proliferation and immortalization of MECs, and may contribute to the suppression of mammary tumorigenesis. While having carried out this study, we have also monitored tumor development in a cohort of Tip30 wild type, heterozygous and homozygous mice for 21 months. We found that one of thirty-one *Tip30*$^{+/-}$ female mice developed mammary ductal adenocarcinoma and one developed mammary liposarcoma. Sixteen of thirty *Tip30*$^{-/-}$ female mice developed lymphoma and other diseases (Tang). The low incidence of mammary tumors in these mice is not surprising given that C57BL6 mice are highly resistant to mammary tumorigenesis (Ullrich 1996). In addition, Tip30 knockout mice often die prematurely due to the development of lymphomas and other diseases thereby possibly reducing the incidence of mammary tumors in *Tip30* knockout mice.

It has been suggested that preneoplastic MECs advance through periods of immortality and hyperplasia before becoming neoplastic (Medina 1993; Medina 2002). The properties of immortality, hyperplasia, and increased neoplastic potential have been demonstrated to be independent of one another. Characterization of four ductal mammary epithelial outgrowth lines by Medina and Kittrell showed these lines to be immortal, but morphologically normal and non-tumorigenic (Medina 1993). Conversely, studies of Ha-*ras*-induced hyperplasias by Miyamoto *et al.*, showed these lesions to be hyperplastic, but not immortal *in vivo* (Miyamoto 1990). *In vitro*, genes such as *c-Myc, ZNF217* and *Bmi-1*, when overexpressed, have been shown to immortalize human MECs (Wang 1998; Nonet 2001; Dimri 2002). As demonstrated here, Tip30 is able to contribute to both the immortality of MECs *in vitro* and hyperplasia of MECs *in vivo*. This could be due, in part, to the ability of Tip30 to repress *c-Myc* transcription. We previously reported that loss of Tip30 results in increased expression of *c-Myc* in murine mammary epithelial cells which could contribute to both the hyperplastic and immortal phenotypes in these MECs (Jiang 2004). Thus, Tip30 is able to contribute to the appearance of two preneoplastic phenotypes, immortality and hyperplasia. It, therefore, may play an important role in the progression of MECs from normal to preneoplastic, and possibly predispose them to tumor formation.

Loss of Tip30 may be a critical step for mammary tumorigenesis.

Tumorigenesis is a multi-step process requiring more than one genetic alteration in a single normal cell to produce neoplastic and metastatic cells. Transformation of cells is an essential step in tumorigenesis which requires specific genetic alterations depending on species and cell types (Rangarajan 2004). The role of oncogenic Ras in this step has been extensively studied. In primary mouse and human fibroblasts it was previously shown that overexpression of oncogenic Ras *in vitro*, could cause a premature senescence (Serrano 1997). Recent work, however, has suggested that early passage primary human

fibroblasts are not susceptible to this Ras-induced senescence, and only after longer periods in culture, when $p16^{INK4a}$ levels have risen, do they become susceptible (Brookes 2002; Benanti 2004). In other cell types, such as human thyroid epithelial cells, oncogenic Ras has been shown to cause prolonged clonal expansion instead of premature senescence (Jones 2000). Ras-overexpressing MECs that are treated with TGF-β1 have been shown to undergo an epithelial to mesenchymal transition (EMT) *in vitro*. EMT is different from scattering, in which cells acquire a fibroblast-like phenotype but do not activate mesenchymal genes as they do in EMT (Jechlinger M. 2003). EMT appears to be an *in vitro* correlate of metastasis, insomuch as cell types that undergo EMT in response to Ras overexpression are able to form tumors and metastases in nude mice, whereas cells that have undergone scattering are only able to form tumors (Janda E. 2002). We show that MECs deficient for Tip30 had a decreased E-cadherin expression and were able to proliferate faster than wild type MECs. Ras-overexpressing *Tip30*$^{-/-}$ MECs, however, were able to proliferate even faster than Ras-overexpressing *Tip30*$^{+/+}$ and control *Tip30*$^{-/-}$ MECs. We observed that the Ras-overexpressing MECs had completely lost expression of E-cadherin and acquired a fibroblastoid morphology (data not shown), suggesting that they may have undergone EMT. Interestingly, Tip30 was first identified as a putative metastasis suppressor in small cell lung carcinomas (Shtivelman 1997). Given that EMT is an *in vitro* correlate of metastasis, this result is consistent with the idea that Tip30 is an important regulator of metastasis, and it may play a role in the proliferation of metastatic cells. Taken together, our results suggest that loss of Tip30, in combination with other genetic alterations, could contribute to the pathogenesis of mammary tumorigenesis.

A possible role of infiltrated lymphocytes in Tip30$^{-/-}$ mammary glands.

It is worthwhile to mention that infiltrated lymphocytes in Tip30$^{-/-}$ mammary glands maybe relevant to tumorigenesis. Evidence suggests that chronic inflammation can play a role in cancer development. Colorectal cancer is more common in patients with ulcerative colitis, a chronic inflammatory disease of the large intestine (Clevers 2004). Also, studies have shown that men who take nonsteroidal anti-inflammatory drugs have a reduced risk of developing prostate cancer or prostate cancer metastases (De Marzo 2003). In both human and mouse mammary glands, it has been shown that preneoplastic and neoplastic mammary tissues contain more lymphocytes than normal mammary glands, and that these cells may actually promote mammary tumorigenesis (Black 1955; Wei 1986; Wei 1986; Wei 1989). Loss of Tip30 results in lymphocyte infiltration in the mammary glands which becomes progressively worse as the mouse ages. Those infiltrating lymphocytes might be responding to preneoplastic changes in Tip30-null mammary epithelial cells such as hyperplasia and immortality. Subsequently, they could over time promote mammary tumorigenesis by releasing factors that influence the proliferation of mammary epithelial cells. The nature of this contribution will be the focus of future studies.

In conclusion, our data provide the first evidence that Tip30 is a new negative factor in the regulation of mammary epithelial cell proliferation and may represent a novel signal pathway in the suppression of breast cancer development. Furthermore, our finding leads us to propose that loss of Tip30 function may occur in human breast cancers and open up new possibilities for developing diagnostic and therapeutic strategies for breast cancer.

Materials and methods

Plasmids and Retroviral infections

Plasmids used for retroviral infections were pBabe-puro and pBabe-puro-RasV12 (a gift provided by Robert Lewis). Viruses were produced by cotransfecting 293T cells with pBabe-puro or pBabe-puro-RasV12 plasmids and helper virus. Supernatant was collected and filtered through a .45μm filter. Early passage MECs were serially infected three times with the virus-containing supernatant and 4μg/mL polybrene at three hour intervals. Cells were selected with puromycin at a concentration of .7μg/mL in MEC media for seven days after infection.

Mice and MECs

The genetic background of *Tip30*$^{+/+}$ and *Tip30*$^{-/-}$ mice was regarded to be identical since they were backcrossed 10 times with C57BL/6 mice or 129SVJ mice, respectively. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Nebraska Medical Center. Primary mammary epithelial cells (MECs) of each genotype were prepared from 8 to 10 week old C57BL/6 mice as previously described (Medina 2000). MECs were maintained in DMEM-F12 supplemented with 2% charcoal-stripped fetal bovine serum, 10μg/mL insulin, 5ng/mL EGF, 1mg/mL BSA, 5μg/mL linoleic acid complex (Medina 2000), 100 units/ml penicillin, 100 μg/ml streptomycin, and 50μg/mL gentamicin (hereafter referred to as MEC media).

Cell culture

Cell growth by trypan blue exclusion. 3000 MECs of each genotype were seeded onto a 96-well plate containing MEC media. Living cells were counted every-other day in triplicate for 5 days using trypan blue dye.

Cell growth by MTT assay. 5000 MECs of each genotype were seeded into a 24-well plate containing MEC media. Cell viability was measured every-other day in triplicate for 5 days using an MTT assay.

Briefly, cells were incubated with .5mg/mL MTT for 3 hours at 37°C. Absorbance was then measured at a wavelength of 584nm using a FLUOstar OPTIMA spectrophotometer.

Population doublings. Population doublings were determined by plating MECs in triplicate in 6-well-plates at a density of $5x10^4$ cells. When cells reached 80% confluency they were counted and replated in new 6-well-plates at a density of $5x10^4$ cells. Population doublings were determined by taking into consideration the number of cells at splitting and the number of times they had been passaged.

Estrous stage determination

Estrous cycle stage was determined by cytological evaluation of vaginal smears. Briefly, the vagina was flushed with 50μL sterile PBS which was then spread on a glass slide. The slides were dried at 37°C and stained using the Protocol™ Hema 3 stain set (122-911, Fisher) according to the manufacturer's instructions.

Mammary transplantation

Ten-week-old wild-type and *Tip30*-null mice were anesthetized with avertin, and mammary gland #4 was removed and cut into small pieces. Small pieces of the removed wild-type and *Tip30*-null mammary glands were transplanted individually into contralateral cleared fat pads of 21-day-old wild-type and *Tip30* knockout mice. Ten weeks or one year after transplantation, the mammary glands were harvested.

Histology and immunohistochemistry

Mammary gland #4 was removed and fixed with 10% buffered formalin and embedded in paraffin blocks. The sections were deparaffinized, rehydrated, and stained with hematoxilin and eosin. For

immunohistochemical staining, unstained sections were rehydrated and incubated overnight at 4°C with antigen-affinity purified anti-Tip30 antibodies (Xiao 1998) and then incubated with anti-rabbit antibodies conjugated with FITC (Vector Lab). After washing with buffers, the same sections were incubated with anti-ERα antibodies conjugated with TRITC (Santa Cruz). After washing, the sections were mounted in 3:1 Vectashield: DAPI (Vector Lab) and analyzed on a Zeiss laser scanner confocal microscope.

BrdU labeling

BrdU labeling analysis was performed in two 8-week virgin mice of each genotype according to the procedure described by the laboratory of Rosen (http://public.bcm.tmc.edu/rosenlab). Mice were injected with BrdU (30mg/kg BW) 2 hours prior to euthanization. Mammary gland #4 was removed and fixed in 4% paraformaldehyde in phosphate buffered saline at 4°C overnight. Paraffin sections were prepared and deparaffinized, hydrated with ethanol, and subjected to microwave antigen retrieval in 10mM citrate buffer, pH 6.0. The sections were first blocked in 5% BSA/0.5% Tween020 in PBS at room temperature for 4 hours, and then incubated with BrdU-FITC antibody (347583, Becton Dickinson) antibody (1:10) at room temperature overnight. After washing, the sections were mounted in 3:1 Vectashield: DAPI (Vector Lab). About 350 epithelial cells from each type of mammary gland were counted for BrdU staining.

Acknowledgements

We thank Drs. C-Wu and Y.Qin for their histopathologic expertise, K. Park for initially performing immunohistochemistry analysis of Tip30 expression in mouse mammary glands, R. Kordum and R. Lewis for providing pBabe-RasH plasmid and J. Wahl for providing E-cadherin antibodies. We also

thank C. Eischen for her generous help and advice. This work was supported by grants from National Institute of Health Grants DK066110-01 and American Cancer Society Grant RSG0216501GMC to H. X. W.T. was supported by Department of Defense postdoctoral traineeship Award, DAMD17-001-0361. J.P. was supported by a NIH graduate student fellowship.

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Figure legends

Figure 1. Tip30 colocalizes with estrogen receptor alpha in murine mammary epithelial cells. Representative pictures of paraffin-embedded mammary gland sections examined by double immunofluorescence for Tip30 and ERα (A) Immunohistochemical staining of Tip30 (green) in wild-type mammary epithelial cells. (B) Immunohistochemical staining of ERα (red) in the same cells. (C) Picture of the two merged signals. Original magnifications are 40X.

Figure 2. Loss of Tip30 results in enlarged ductal lumens and ductal hyperplasia. Whole mount analysis of mammary glands from *Tip30*$^{+/+}$ (A) and *Tip30*$^{-/-}$ (B) female mice. After whole mount of mammary glands was pictured, the tissues were kept in Xylene overnight and paraffin-embedded for sectioning. The sections were then stained with hematoxylin. The largest lumen seen in the *Tip30*$^{+/+}$ mammary gland section (C). An example of the *Tip30*$^{-/-}$ducts (D). H & E staining of ducts from *Tip30*$^{+/+}$ (E and G) and *Tip30*$^{-/-}$ (F and H) mammary glands. All mammary glands pictured are from virgin female mice at the age of 8 weeks. Original magnification in A and B, 4X; in C and D 20X; in E-H, 40X.

Figure 3. *Tip30*-null mice have an increased incidence of alveolar buds in the mammary glands. All mammary glands were prepared from 1-year-old, virgin female mice in diestrus. (A) Whole mount analysis of mammary glands from C57BL6, 129SV, and FVB/C57BL6 *Tip30*$^{+/+}$ and *Tip30*$^{-/-}$ mice. (B) Percentage of DB and AB in the mammary glands of 1-year-old female mice in diestrus. Stars indicate a p value of $\leq .05$

as determined by student's t test. DB = ductal branching, AB = alveolar bud. (C) H & E stained sections of mammary glands from *Tip30*$^{+/+}$ and *Tip30*$^{-/-}$ female mice.

Figure 4. The effects of Tip30 on the mammary gland are intrinsic. Histological analysis of *Tip30*$^{+/+}$ and *Tip30*$^{-/-}$ mammary glands 10 weeks after transplantation into Tip30$^{+/+}$ (A) and *Tip30*$^{-/-}$ (B) recipients. (C) Whole mounts of transplanted *Tip30*$^{+/+}$ and *Tip30*$^{-/-}$ mammary glands 1 year post-transplantation into wild-type recipients. (D) Whole mount image of infiltrating lymphocytes observed in *Tip30*$^{+/+}$ and *Tip30*$^{-/-}$ mammary glands 1 year post-transplantation into wild-type recipients. (E) H & E stained sections of infiltrating lymphocytes observed in *Tip30*$^{+/+}$ and *Tip30*$^{-/-}$ mammary glands 1 year post-transplantation into wild-type recipients. After whole mount of mammary glands were prepared and pictured, the tissues were kept in Xylene overnight and paraffin-embedded for sectioning.

Figure 5. Loss of Tip30 increases proliferation of mammary epithelial cells. (A) BrdU labeling and DAPI staining of wild-type and *Tip30*-null mammary glands from virgin female mice at the age of 8 weeks. (B) Cell counts of MECs using trypan blue dye (left panel) and MTT assay of MECs (right panel). All data points represent three independent samples. (C) Cell counts of Ras V12-infected MECs using trypan blue dye (left panel) and MTT assay of Ras V12-infected MECs (right panel). All data points represent three independent samples.

Figure 6. Tip30-null MECs rapidly immortalize *in vitro*. Population doubling curve for *Tip30*$^{+/+}$ and *Tip30*$^{-/-}$ MECs *in vitro*. Viable cells were counted every three days then re-plated on dishes. The results represent the mean of three independent experiments. Similar results were obtained from three pairs of *Tip30*$^{+/+}$ and *Tip30*$^{-/-}$ mice. (A) Population doublings through day 41. (B) Population doublings through day 141. (C) Western blot analysis of E-cadherin from pre-crisis *Tip30*$^{+/+}$ and *Tip30*$^{-/-}$ MECs; and post-crisis *Tip30*$^{-/-}$ MECs. Beta-actin was used as a control.

Table 1. Loss of Tip30 results in an increased incidence of lymphocyte infiltrates in the mammary glands. After whole mount analysis of mammary glands, the number of lymphocyte infiltrations was counted in each mammary gland from *Tip30*$^{+/+}$ and *Tip30*$^{-/-}$ mice (n=6) under a low power microscope. First two columns represent mammary glands harvested 1-year post-transplant from wild-type recipients. Second two columns represent mammary glands from untransplanted mice.

Fig. 1

Fig. 2

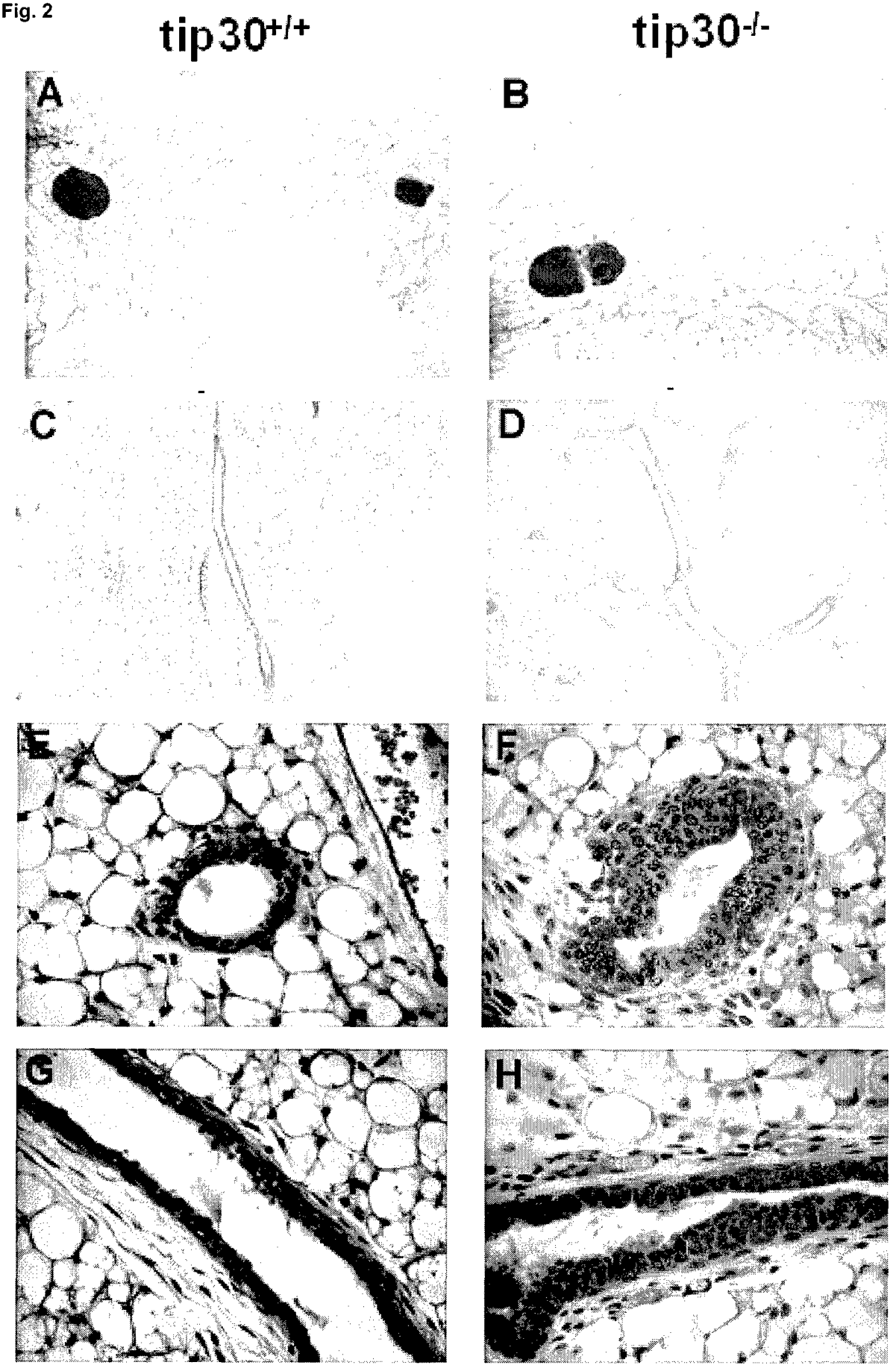
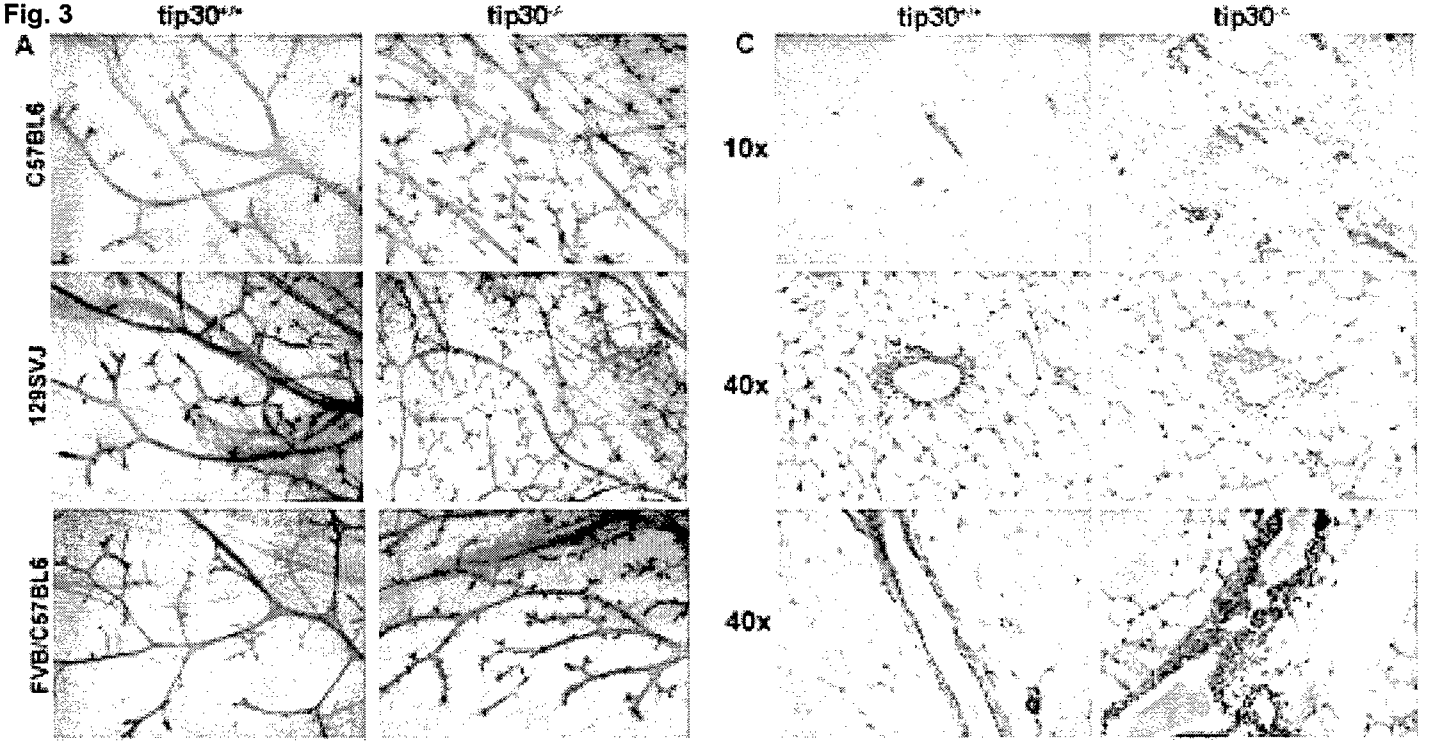


Fig. 3



B Alveolar bud formation in 1-year-old tip30 females

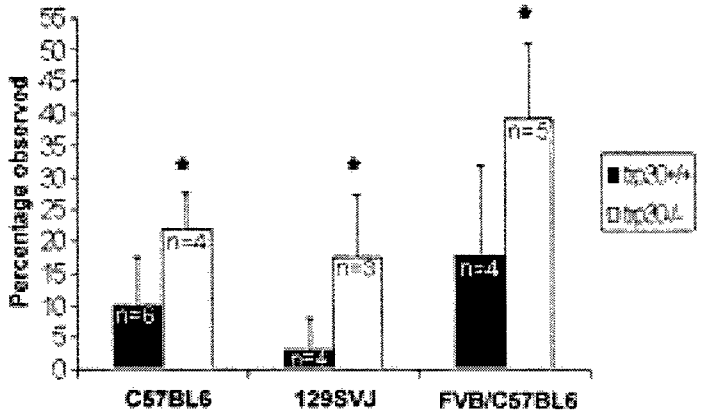


Fig. 4

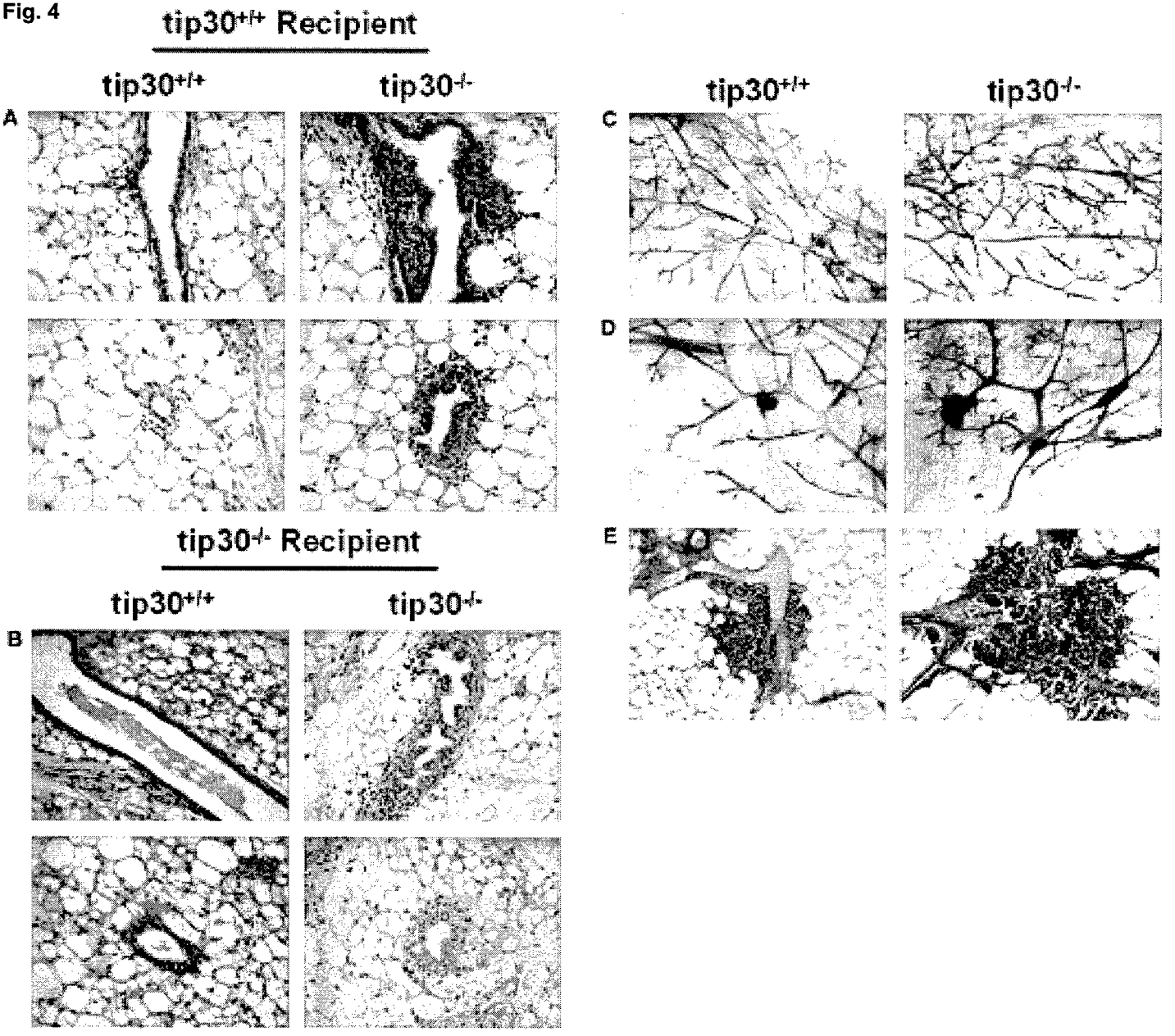

tip30+/+ Recipient
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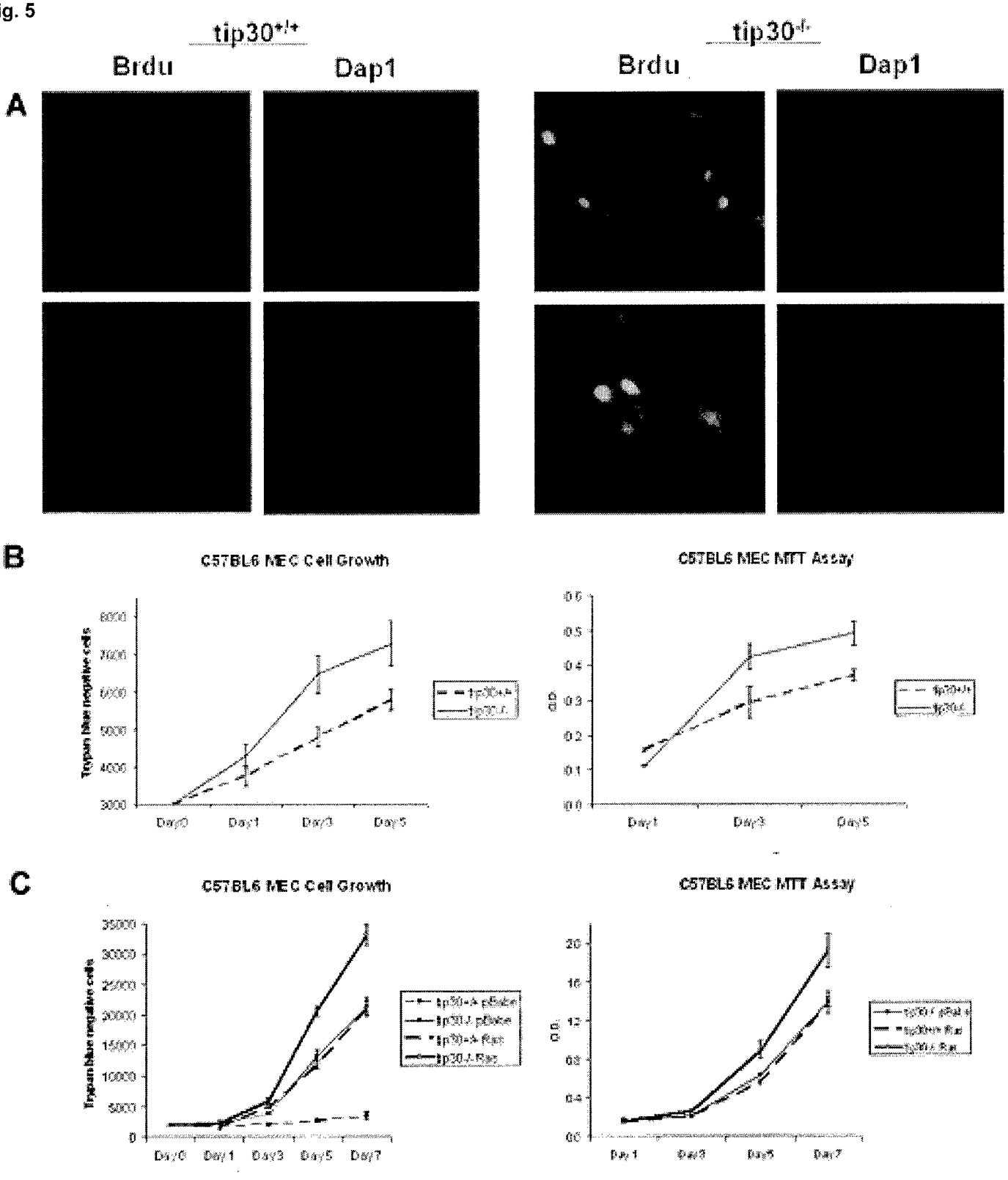
Fig. 5


A
tip30+/+
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Trypan blue negative cells
Day0
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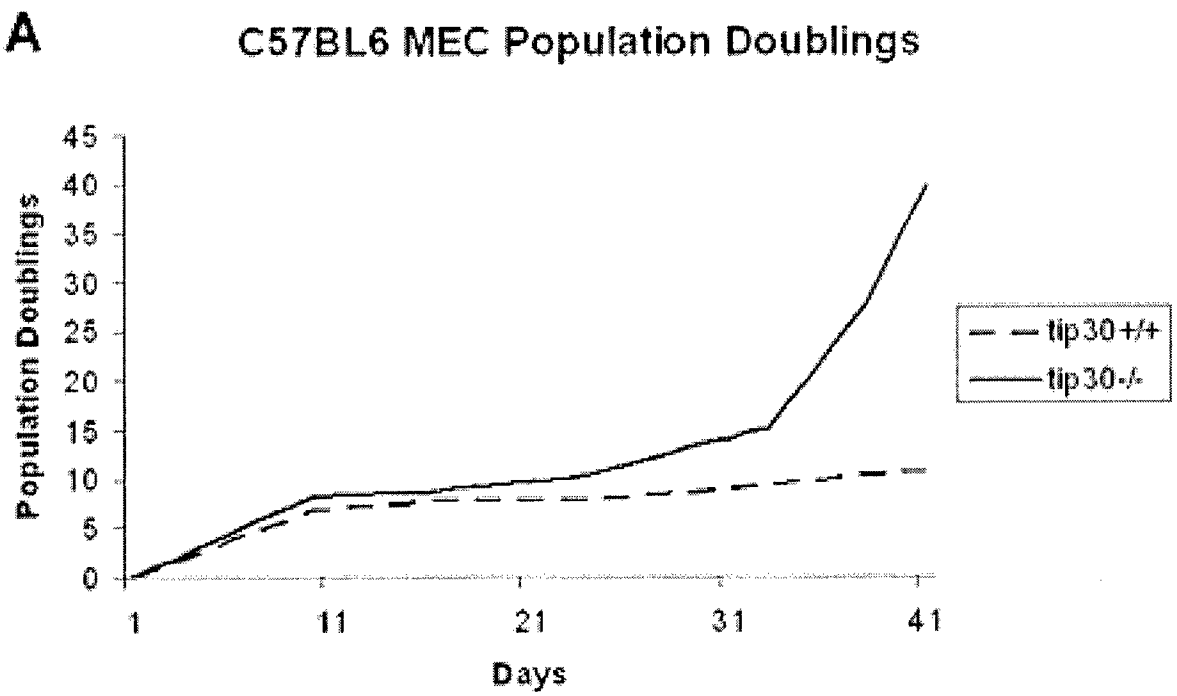
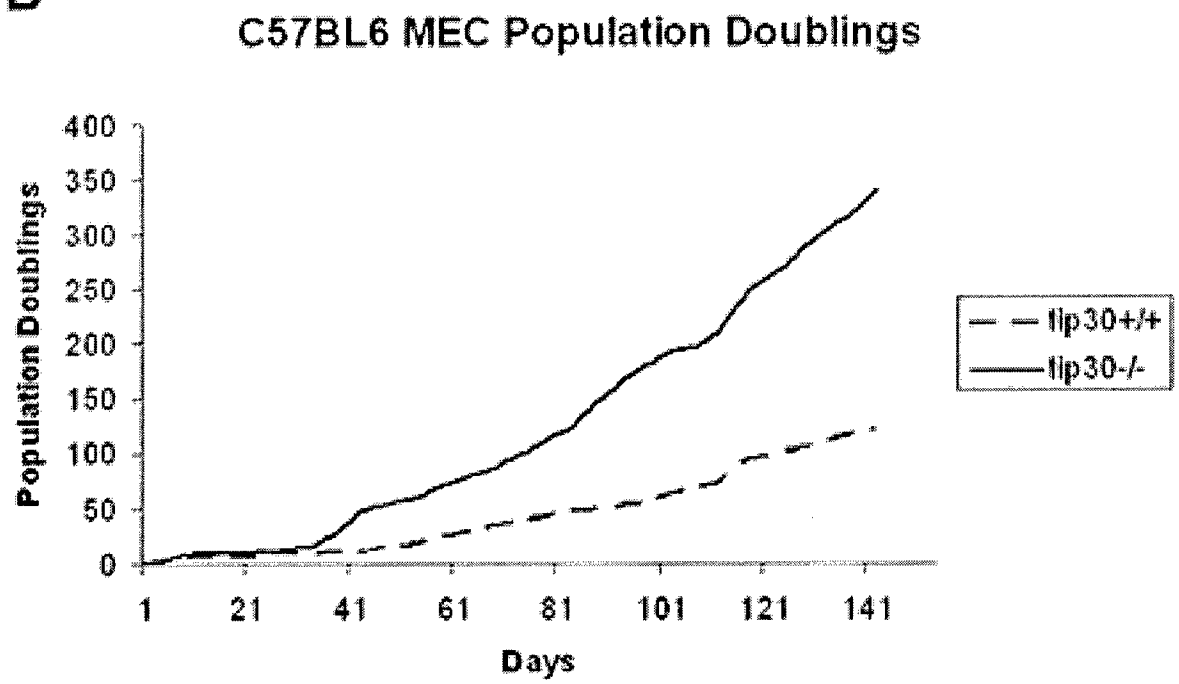
Fig. 6

A


C57BL6 MEC Population Doublings
Population Doublings
45
40
35
30
25
20
15
10
5
0
1
11
21
31
41
Days
tip30+/+
tip30-/-

B


C57BL6 MEC Population Doublings
Population Doublings
400
350
300
250
200
150
100
50
0
1
21
41
61
81
101
121
141
Days
tip30+/+
tip30-/-

C

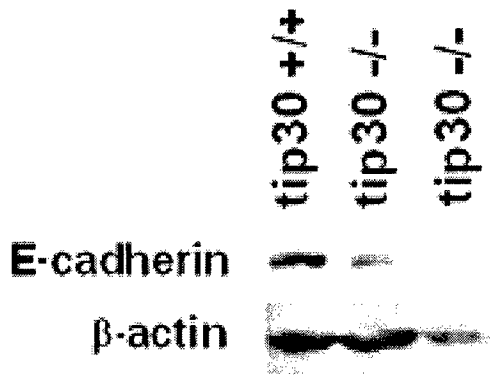

tip30 +/+
tip30 -/-
tip30 -/-
E-cadherin
β-actin

Table 1	Transplant		No Transplant	
Mouse	**tip30+/+**	**tip30-/-**	**tip30+/+**	**tip30-/-**
#1	0	0	0	0
#2	0	0	0	0
#3	1	4	0	1
#4	1	4	0	3
#5	1	8	0	4
#6	0	9	0	6
Average # per gland:	0.5	4.2	0.0	2.3

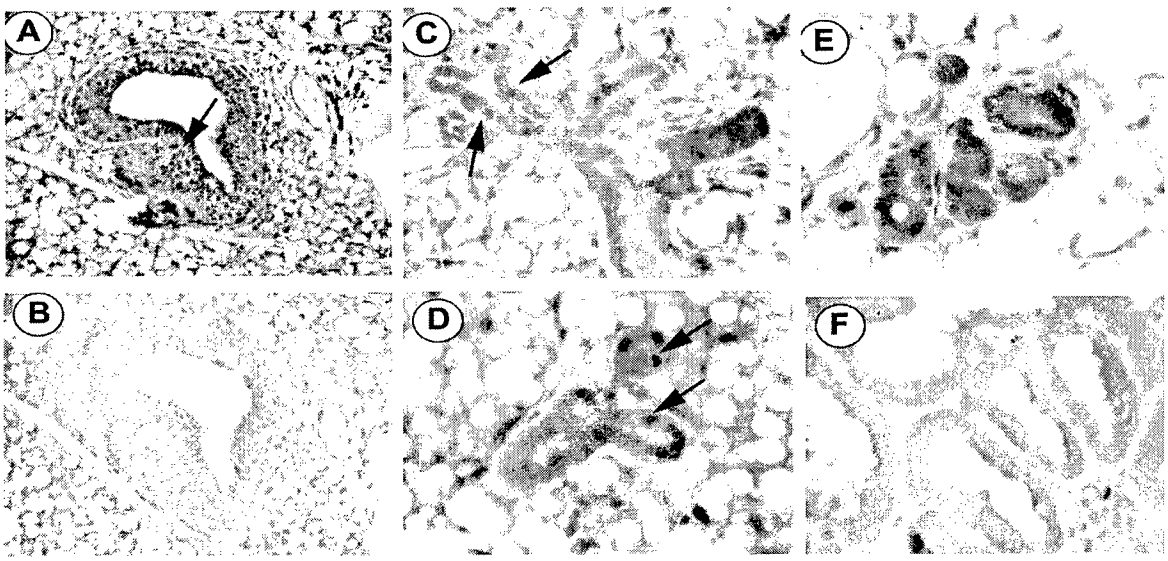
Supplemental data

To further investigate the role of Tip30 in mammary gland development, we established a developmental profile of Tip30 expression in the murine mammary gland by immunohistochemical analysis and then analyzed the phenotypic consequences of Tip30 deficiency on mammogenesis in genetically engineered mice. Staining of paraffin sections of mammary glands from five-week-old virgin mice with anti-Tip30 serum showed that Tip30 is specifically expressed in the luminal and basal compartment of terminal end buds (TEBs) (Fig. 1 A). The nuclear staining appeared noticeably stronger in the luminal compartment of the TEB. Moreover, staining in the stroma is likely nonspecific since it was also observed when this tissue was stained with the pre-immune serum (Fig. 1B). No specific staining, nuclear or cytoplasmic, was observed in epithelial cells on the control slides with the pre-immune serum (Fig.1B). In mature virgin mice, Tip30 staining was again noted in the nucleus (Fig. 1C and D) and cytoplasm of ductal epithelial cells. At day 12.5 of pregnancy, Tip30 was localized mainly in the cytoplasm of highly proliferating alveolar cells (Fig. 1 E), whereas Tip30 remained in the nuclei in many adjacent ductal cells of tertiary branches (data not shown). During lactation, Tip30 was exclusively expressed in the cytoplasm of terminally-differentiated epithelial cells (Fig. 1F). These results suggest that Tip30 expression is developmentally regulated in mammary epithelial cells.

Figure Legend

Figure 1. Tip30 expression is developmentally regulated in mammary epithelial cells. Tip30 expression in murine mammary glands. Murine mammary glands were

isolated from various development stages. Formalin fixed, paraffin-embedded sections were immunoblotted with an anti-Tip30 serum (A, C-F) and pre-immune serum (B) and counter-stained with hematoxylin. (A) 5-week old virgin. (B) 5-week old virgin stained with pre-immune serum as a control. (C and D) Mature virgin. (E) Pregnancy. (F) Lactation. Arrows indicate nuclear staining of Tip30.


A
C
E
B
D
F